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Development of an Integrated Process for Continuous Winemaking

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I wish to dedicate this work to the three persons in my life that never stopped their will of learning and improving themselves: to my mother Nezi, my grandfather Georgi and my love Rui. You are an example to follow.

LIST OF PUBLICATIONS

This thesis is based on the following original articles:

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Genisheva, Z., Mota, A., Mussatto, S., Teixeira, J.A. Oliveira, J.M. Integrated continuous winemaking process involving sequencial alcoholic and malolactic fermentations.

SUMMARY

Microorganisms play an important role in the food industry, particularly in the processing of foods and beverages. In wine production, two consecutive fermentation processes occurs, involving yeasts (alcoholic fermentation) and lactic acid bacteria (malolactic fermentation), its control is being crucial to obtain high quality products. Considering that the raw material used in wine production is seasonal these two fermentations are conducted in batch mode. The advances made in cell immobilization techniques in the last decade, led to further exploration of these techniques for continuous fermentation processes.

This work aims at the implementation of a continuous wine production process by the use of sequential continuous fermentations with immobilized cells, in the different stages of the fermentative process of winemaking, *i.e*. in alcoholic fermentation and in malolactic fermentation. For this purpose, separate immobilization of commercial strains of *Saccharomyces cerevisiae* and *Oenococcus oeni* were carried out by adsorption on different natural waste materials such as: grape skins, grape stems, grape seeds and corn cobs. Following preliminary batch trials the best immobilized cell systems were studied in the corresponding packed bed reactors operated in continuous mode. The influence of the operating parameters of the system over the cell physiology and quality of the final product - physicochemical and sensory characteristics (colour, aroma and taste) - was evaluated. The operational stability of the immobilized systems was also characterized.

Fermentations with immobilized cells were much faster than fermentations with free cells and the quality of the product was maintained, making the process more advantageous and economical. Also, the uses of natural supports that are agro-industrial residues make the process more environmentally friendly. The quality of the wines produced using immobilized cells was confirmed by sensory evaluation as it was difficult to distinguish between wines produced by the different methods. Volatile aroma compounds evaluation confirmed the good quality of wines produced with immobilized cells. Initially, wines produced with immobilized cells presented a darker colour compared with wines produced by the traditional free cells method. However, with time and with the reuse of the immobilized cell system the colour stabilized and wines became similar to the wines produced with free cells. The used immobilized cell systems were found to have strong operational and mechanical stabilities. Moreover it was found that immobilized cells can be stored for at least 1 month at 4 °C and used again in fermentation processes of winemaking, as confirmed by its successful application in continuous alcoholic and malolactic fermentation processes. Overall, the integrated continuous process of winemaking demonstrated to have good operational stability making it a valuable alternative for the production of good quality wines.

RESUMO

Os microrganismos desempenham um papel relevante na indústria alimentar, nomeadamente no processamento de alimentos e de bebidas. No caso particular da produção de vinho, ocorrem dois processos fermentativos, onde intervêm leveduras (fermentação alcoólica) e bactérias lácticas (fermentação maloláctica), cujo controlo é crucial para obtenção de produtos de qualidade. Devido à sazonalidade da matéria-prima, estas duas fermentações são tradicionalmente conduzidas em descontínuo. Nos últimos, os avanços conseguidos nas técnicas de imobilização celular e o uso de suportes passíveis de contacto com produtos alimentares, conduziu a uma maior exploração de processos fermentativos em contínuo.

Este trabalho teve por finalidade a implementação, em contínuo, com células imobilizadas, das etapas fermentativas de um processo de vinificação, *i.e.* a fermentação alcoólica e a fermentação maloláctica. Para o efeito foi ensaiada a imobilização de culturas comerciais de *Saccharomyces cerevisiae* e de *Oenococcus oeni*, por adsorção natural em suportes resultantes de resíduos da indústria agroalimentar: películas de uva, bagaço de uva e carolo de milho. Após operação em modo descontínuo as melhores combinações célula-suporte foram estudadas em reator empacotado. A qualidade final do produto, quer em termos físico-químicos quer em termos organolépticos (aspeto, gosto e aroma), depende não só da qualidade da uva utilizada, que depende da casta, do *terroir* e do ano de vindima, mas também dos procedimentos adotados durante todo o processo de vinificação. Sendo assim foi avaliada a influência dos parâmetros de operação do sistema sobre a fisiologia celular e a qualidade do produto final. Foi também avaliada a estabilidade operacional dos sistemas imobilizados.

As fermentações conduzidas com células imobilizadas foram muito mais rápidas do que fermentações com células livres, tornando o processo mais vantajoso e económico. Assim também o uso dos resíduos agroindustriais torna o processo mais ambientalmente amigável. A avaliação sensorial dos vinhos produzidos com células imobilizadas e com células livres demonstrou que é difícil distingui-los. A avaliação aromática dos vinhos elaborados com células imobilizadas demonstrou que estes vinhos são de boa qualidade. Inicialmente os vinhos produzidos com células imobilizadas apresentaram uma cor mais escura comparados com os vinhos produzidos pelo método tradicional com células livres. No entanto, com o tempo e com a reutilização dos sistemas imobilizadas a cor tende a estabilizar e torna-se semelhante aos dos vinhos produzidos com células livres. Os sistemas de células imobilizadas evidenciaram estabilidades operacionais e mecânicas. Além disso, verificou-se que as células imobilizadas podem ser armazenadas durante pelo menos 1 mês a 4 °C e utilizadas novamente nos processos fermentativos de vinificação. Os processos de fermentação em contínuo (alcoólica e maloláctica) com células imobilizadas, foram bem sucedidos e bem conduzidos. O processo integrado de vinificação em contínuo demonstrou uma estabilidade operacional eficaz. Os resultados obtidos foram considerados promissores para fomentar estudos futuros.

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Variables and Constants

$\bar{C}^*$	saturation of wines produced by free cells
$\bar{L}$	lightness of wines produced by free cells
C^*	colour saturation
$C_{ac.ac}$	concentration of acetic acid
$C_{et.}$	ethanol concentration
$C_{lac.ac}$	concentration of lactic acid
$C_{mal.ac}$	concentration of malic acid
C_x^*	saturation of wines produced by immobilized cells
D	dilution rate
D_{im}	immobilized dead cells
F	relative frequency
GM	geometric mean
I	relative intensity
L^*	lightness of colour
L_x^*	lightness of wines produced by immobilized cells
Q_P	productivity
RCF	relative centrifuge force
sd	standard deviation
t	fermentation time
TA	total acidity, expressed as tartaric acid
$X_{f.cel}$	free cell concentration
X_i	concentration of immobilized cells in the assay
X_{im}	Immobilized cell
X_t	total produced cells
Y_i	immobilization efficiency
$Y_{P/S}$	product yield
$Y_{X/S}$	cell yield factor

Acronyms

AF	alcoholic fermentation
B	batch assay
BC	Before Christ
FC	free cell assay
GC-FID	gas chromatography with flame ionization detector
GC-MS	gas chromatography mass spectrometry
HPLC	high pressure liquid chromatography
ICS	immobilized cell systems
IS	internal standard
MFBT	multistage fixed bed tower reactor
MLF	malolactic fermentation
OIV	International organization of vine and wine
PB	packed bed reactor
QDA	Quantitative Descriptive Analysis
SEM	scanning electron microscopy
T	treated
U	untreated

Variables and Constants with Greek letters

$\eta_{mal.ac}$	efficiency of malic acid degradation
a^*	colour parameter

b^*	colour parameter
ΔC^*	variation in colour saturation
ΔL^*	variation in lightness of colour

Latin expressions

i.e.	*id est* (this is)
et al.	*et alii* (and others)
e.g.	*exempli gratia* (for example)

1. Motivations and Outline

This chapter introduces the main topics of the thesis and its main goals. The outline of the thesis is also presented.

1.1 Scopes and objectives

Nowadays, the major challenges in the food industry are the development of healthier, safer and environmental friendly foods. In addition, for the achievement of these objectives is essential to develop advanced production technologies to make the food production processes more economically attractive. The use of immobilized cell systems is a technology that has been widely applied in the production of several products such as bio-ethanol. However, in food industry, as in beer production, the technology is only been studied to prove its applicability. There are also few studies developed for the production of wine using this innovative fermentation system aiming to overcome the associated technical limitations such as the product quality issues, operational costs associated with material used as support of the cells, as well as the immobilization process.

The main objective of this work was the development of an integrated continuous process of winemaking with immobilized cells.

The main focus areas were:

- selection of a suitable support for cell immobilization that can be easily accepted by the consumer, cheap and abundant in the nature;
- application of the immobilized cells system for the alcoholic and malolactic batch fermentations in winemaking;
- application of cells immobilized in supports in continuous alcoholic and malolactic fermentation processes in winemaking;
- integration of alcoholic and malolactic fermentations in a continuous winemaking process;
- study of the stability and operational issues of the continuous winemaking process and strategies to store the immobilizing support;
- characterization of the wine produced by the immobilized cells production system.

1.2 Overview

Wine is a well-known ancient beverage spread al over the world. It had an important role in the old civilizations and reached our days with no less importance. The grapes were used in the ancient times, as confirmed by the finding of an installation for winemaking in the territory of Armenia dating to around 4000 BC. Eastern Europe is considered to be the birthplace of the vine, more specifically the area between and below the Black Sea and the Caspian Sea. In 2011

according to statistics of the *International Organisation of Vine and Wine*[1] (OIV, 2012), 7.6×10^6 ha of vines, allowed the production of 265×10^8 L of wine around the world. This amount is changing every year, depending on various circumstances like occasionally unfavorable climate conditions

The two main processes associated with wine production are the alcoholic and malolactic fermentations. Traditionally, the wine fermentation technology uses free yeast biomass suspended into the must that ferments in an unstirred batch reactor during long periods of time, making the fermentation a very time-consuming stage of the process. In the last decades new methods have been under study, in order to improve the fermentation performance and productivity, namely the use of immobilized yeast cells which speed up the fermentation process. By doing so, labor requirements are diminished, thus simplifying time-consuming procedures which can help to reduce costs. Continuous winemaking technology with immobilized cells is still under study to demonstrate its application in industrial processes. However, its economic benefits are the basis of a research area aimed at studying and implementing continuous fermenters.

1.3 Outline of the thesis

The present thesis was divided in eight chapters according to its main objectives:

In the current **Chapter 1** are presented, in short, the motivation and the main objectives of this thesis.

In the following **Chapter 2** is presented a general introduction to the subject of winemaking by the traditional method and by immobilized cell systems. An overview of the previous studies developed in the wine production with immobilized cells is done.

According to the main objectives of this thesis, the obtained **results** are presented from **Chapter 3** to **Chapter 7**. Each of these chapters is divided in *introduction*, *materials and methods*, *results and discussion* and *conclusions*.

The main goals of **Chapter 3** are the search and selection of adequate natural materials to be used as support for the immobilization of yeast cells. Different supports as well as different supports treatments were evaluated.

In **Chapter 4**, the support that gave better results in the previous Chapter 3 was used yeast cells immobilization and applied in alcoholic fermentation of wine. Studies on the operational activity and stability, as well as the possibility of storage of the immobilized cell systems are

[1] *www.OIV.int* 22 October of 2012

also presented. A comparison between the wines produced with immobilized cells and traditionally produced wines is also done.

The main solid waste of the wine industry is called grape pomace. In **Chapter 5** grape pomace was studied as a potential support for yeast cell immobilization and after cell immobilization used for winemaking. The produced wines were analyzed and compared to traditionally produced wines.

In **Chapter 6** the supports that gave better results in Chapter 3, were studied as a possible support material for immobilization of bacterial cells. The operational stability and activity of the immobilized cell systems was studied during malolactic fermentation. The inhibitory effect of some compounds on the bacterial activity was assessed. Finally the possibility of storage of the immobilized cell system was evaluated.

In **Chapter 7**, in agreement with the overall objective of this thesis, the immobilized yeast and bacteria cells were used in continuous alcoholic and malolactic fermentations and the possibility of an integrated continuous winemaking process was studied. The produced wines were analyzed.

Chapter 8 accounts for the final conclusions of this work, as well as the possibility of future research activities.

2. General Introduction

In this chapter the focus will be on the introduction to the subject of this thesis, starting by describing the different stages of the traditional winemaking process. The outcome of the use of immobilized cell systems is presented, including the description of the types of supports usually applied. Biological reactors with immobilized cell systems operating in continuous mode are also addressed.

2.1 Traditional winemaking and wine aroma

Traditionally, the process of winemaking includes several steps. The first stage of the wine production is related with the preparation of the grapes juice – must – which includes: harvesting of grapes, crushing, maceration (in the case of red wines), pressing and must clarification. When the must is ready, the next phase begins where the conversion of sugar rich liquid into ethanol – malo-alcoholic/alcoholic fermentation – occurs, followed by malolactic fermentation (if desired), maturation, stabilization, bottling and bottle fermentation (in sparkling wine production).The introduction of the immobilization technology in the wine making can be considered at the primary and secondary fermentation steps of winemaking (Figure 2.1).

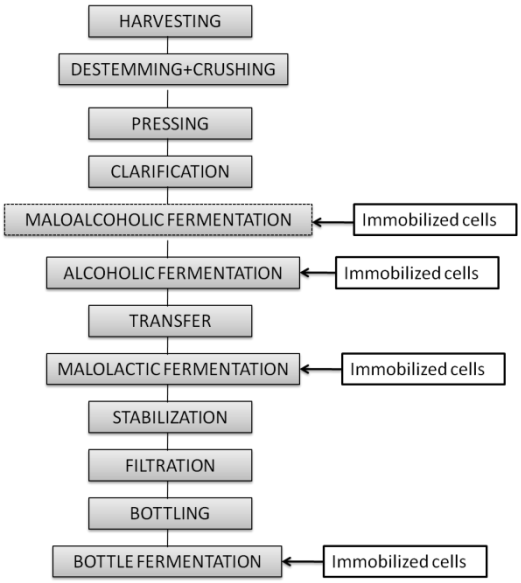

Figure 2.1.Winemaking technology and the possibility of using immobilized cells.

The two more important and critical stages of the conversion of grape must into wine are: Alcoholic Fermentation and Malolactic Fermentation. The details about those fermentations are described below. In some cases, before the alcoholic fermentation is desirable a deacidification of the grape must by means of so called malo-alcoholic fermentation. During the malo-alcoholic fermentation malic acid is transformed into ethanol, thus decreasing the acidity of the grape must. However, malo-alcoholic fermentation is used mostly in laboratory studies and not in traditional winemaking.

2.1.1 Alcoholic fermentation

Alcoholic fermentation (AF) is the primary fermentation during winemaking. Throughout the AF the sugars of the must, mainly glucose and fructose, are transformed to ethanol and carbon dioxide, according to the following equation:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2 \qquad \text{(eq. 1)}$$

The fermentation process is much more complex than this simplified equation and several other compounds are formed during different chemical and biochemical iterations along the fermentation evolution. The main compounds formed during the alcoholic fermentation are ethanol and glycerol. Other compounds, in much lower concentrations, are also formed, contributing for the global tase and aroma of the wine like: organic acids, higher alcohols, volatile fatty acids and their esters and aldehydes (Oliveira, 2000). The volatile compounds formed at this step represent the biggest contribution to the wine aroma (Oliveira, 2000).

Traditionally, the fermentation of the must starts spontaneously by the yeast that naturally covers the surface of the grapes. Most of the strains of that yeast biomass are not tolerant to ethanol and for this reason, during the natural fermentation, there is a succession of organisms that prevails troughout the process. According to Hornsey (2007) the succession of principal type of yeasts prevailing during spontaneous fermentation is as follows:

Kloeckera spp. → *Hansenula* spp. → *Saccharomyces cerevisiae* → *Saccharomyces bayanus*

Even thought, *S. cerevisiae* is present on the grapes and in the fresh must in low percentages, it is considered to be the principal "fermenting" yeast during AF (Swiegers *et al*., 2005). Based on this fact, the AF conducted in this work was performed by *S. cerevisiae*. Moreover, the selected commercial specie *QA23* (*Lalvin,* Proenol) is a yeast isolated from the region of *Vinhos Verdes* – local Appellation of Origin wine region.

2.1.2 Malolactic fermentation

The malolactic fermentation (MLF) is a secondary fermentation in which L-malic acid produced along the AF is transformed in L-lactic acid and carbon dioxide. In summary, the process can be explained with the simplified equation:

$$C_4H_6O_5 \rightarrow C_3H_6O_3 + CO_2 \qquad \text{(eq. 2)}$$

Normally, MLF occurs after the alcoholic fermentation. The main consequence of the MLF is the decreasing of the wine acidity, modifying the aroma of wine to a subtle form (Oliveira, 2000), improving the character of the wine as well as intensifying the wine bouquet (Ribéreau-Gayon *et al*., 2006). The MLF brings microbiological stability to the wines by reduction of its acidity and increasing the pH. White wines are more sensitive to the changes caused by the MLF (Ribéreau-Gayon *et al*., 2006). In terms of acid conversion, the fermentation of 1 g of malic acid per litre reduces the total acidity, expressed as tartaric acid, by approximately 0.6 g/L (Ribéreau-Gayon *et al*., 2006).

Normally, MLF starts when AF has finished and involves the growth of particular lactic acid bacteria such as: *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus* (Maicas, 2001). MLF is a time consuming and difficult to control process. It is strongly influenced by environmental conditions and the process is often extended in time, or in the worst scenario, it can fail completely (Maicas, 2001). *Oenococcus oeni* is the main bacterial specie found in wine during MLF once it is the most adapted to high concentrations of ethanol and low pH values (Ribéreau-Gayon *et al*., 2006). Based on this fact, in this work the MLF was conducted by a commercial strain of *Oenococcus oeni* (Uvaferm® Alpha, Lallemand). The ability for spontaneous MLF is dependent on the grape region, vineyard and year. The performance of MLF depends on environmental conditions such as pH, temperature, ethanol, nutrients, sulfur dioxide and wine flora.

2.1.3 Aroma compounds of wine

The aroma is one of the most important characteristic of the wine and together with the flavour defines its distinctive characteristics (Swiegers *et al*., 2005). The aroma of wine is a detector for its quality and influences the consumer preferences. The main constituents of the wine aroma are the present volatile compounds. The volatile compounds stimulate the sensorial organs that are responsible for the olfaction. The volatile compounds can reach the receptors of the olfactory epithelium by two ways: directly through the nose – orthonasal route, or through the mouth – retronasal route. The intensity of an olfactory sensation depends not only on the concentration of the component in the liquid phase but also on its volatility, its vapour pressure and its olfactory perception threshold (Meilgaard *et al*., 1975). The olfactory perception threshold could be defined as the smallest stimulus capable of producing an olfactory sensation in at least 50 % of a jury of a sensory panel.

According to the origin and considering the biotechnological sequence of winemaking, wine aroma can be classified into four different groups (Bayonove *et al*., 1998): I) varietal aroma, typical of the grape variety that directly passes through the must, depending essentially on soil, climate, phytotechny, sanitary conditions and degree of ripeness; II) pre-fermentative aroma, originates during grape processing and the subsequent operations (the varietal and pre-fermentative aromas are also called the primary aroma); III) fermentative aroma (the secondary aroma), produced by yeast during alcoholic fermentation and by lactic acid bacteria during malolactic fermentation and IV) post-fermentative aroma (the tertiary aroma), resulting from the transformations that occur during the conservation and aging of wine. The combination of different volatile compounds such as alcohols, esters, organic acids, aldehydes, ketones, terpenes, among others, form the character of the wine, allowing differentiating between wines (García-Jares *et al*., 1995).

Varietal aroma of wine includes volatile compounds belonging to one of the following groups: monoterpenols, methoxypyrazines, C_{13}-norisoprenoid, volatile phenols and dimethyl sulphide (Vilanova and Oliveira, 2012). Except for the methoxypyrazines, these compounds occur in grapes in the form of non-volatile precursors like unsaturated fatty acids, glycosides, carotenoids, cysteine S-conjugates and phenolic acids, which can originate flavour compounds during or after the technological sequence of winemaking (Bayonove *et al*., 1998). However, monoterpenols are also abundant as free odorants in some grape varieties.

Pre-fermentative compounds are formed during harvesting, transport, crushing, pressing, clarification as well as during eventual must heating or grape maceration (Bayonove *et al*., 1998). The compounds involved in the pre-fermentation aroma are aldehydes and alcohols with 6 carbon atoms (C_6-compounds). The C_6-compounds derive from grape lipids (linoleic and linolenic acids), in the presence of oxygen, by a sequence of enzymatic reactions (Crouzet *et al*., 1998).

Fermentative compounds are produced mainly during the alcoholic fermentation of wines and in a minor part, but no less important, during malolactic fermentation, if occurred. The production of fermentative compounds depends mainly on fermentation temperatures and microorganism species. Fermentative compounds are alcohols, fatty acids, esters, organic acids, and some volatile phenols (Bayonove *et al*., 1998). Fermentative compounds present the major quantity of volatile compounds in wines and contribute for its vinous character.

Post-fermentative aroma compounds are also known as the *bouquet*. These compounds are formed during storage and aging of wine by chemical changes in its volatile composition.

2.2 Immobilization methods

Cells of biomass can be kept inside of bioreactors in several ways. There are four main immobilization techniques for yeast cells: attachment to a surface, entrapment within a porous matrix, cell aggregation (flocculation) and containment behind barriers (Kourkoutas *et al*., 2004; Verbelen *et al*., 2006).

The attachment to a surface can be done by natural adsorption, electrostatic forces or covalent binding, with cross-linking agents. Van der Waals forces, electrostatic interactions and covalent bonding, have an important part in the adsorption process (Margaritis and Kilonzo, 2005). The attachment of cells to an organic or inorganic support may be obtained also by creating chemical bonds (covalent) between cells and the support using cross-linking agents. However this immobilization procedure is generally incompatible with cell viability, since the cross-linking agents are highly toxic for the microbial cells decreasing their activity (Junter and Jouenne, 2004; Strehaiano *et al*., 2006). As consequence, this method of immobilization is no

longer used for microbial cells but still remains suitable for the immobilization of enzymes (Strehaiano *et al*., 2006). The adsorption of cells on different types of support is a natural process. The surface of the immobilization support is important in the process of adsorption of cells as rough surfaces allows the cell retention into the support´s cavities (Brányik *et al*., 2004a; Genisheva *et al*., 2011). This immobilization technique is often used as it is an easy and natural process that takes place spontaneously. However, there is no barrier between the liquid and the immobilized cell and the cells can be easily detached from the support. Normally, the equilibrium between free and immobilized cells is established at some point of the cell growth. The detachment of cells depends on the age of the cell, cellular wall composition, pH and ionic composition of the medium. However, the desorption is compensated with the growth of new cells on the support (Strehaiano *et al*., 2006). The natural adsorption technique is advantageous over other types of immobilization as the oxygen transfer is good and no scale-up drawback exists (Ory *et al*., 2004). In the last years, natural adsorption is the most used technique for yeast cell immobilization and further applied in winemaking (Kandylis *et al*., 2010, 2012a and 2012b; Torresi *et al*., 2011; Tsakiris et al., 2006). According to this tendency, as well as the time and cost savings, and mostly the simplicity of the adsorption method, this technique was used in the present work for the immobilization of yeast and bacteria cells.

The second most important technique of immobilization is the entrapment within a porous matrix (Verbelen *et al*., 2006). It can be performed by two approaches: a) the cells are introduced in a porous material and, after growing, their mobility is restricted by the presence of other cells and by the matrix; b) a solid matrix is synthesized *in situ* around the cells. The cells are incorporated in the matrix of a more or less rigid polymer. The polymers are synthetic such as polyacrylamide, or can be made from proteins (gelatine, collagens) and polysaccharides (cellulose, alginate, agar, carrageenan). This technique can be expensive and time consuming (Verbelen *et al*., 2006), with serious drawbacks such as diffusion limitations of nutrients, metabolites and oxygen, as well as instability of the gel beads and detachment of cells (Kourkoutas *et al*., 2004). However, for compounds with molecular weight of less than 5 kDa the diffusion problem is not a leading factor (Strehaiano *et al*., 2006). Calcium alginate gel is the most commonly used material for cell entrapment in the food industry (Strehaiano *et al*., 2006). The cells on the surfaces of the alginate beads can be released from the beads and because of this fact, it was proposed in the 80's to make an external layer of sterile alginate and produce double layer alginate beads.

Containment behind a barrier can be achieved by two main methods: entrapment of the cells in microcapsules and by the use of microporous membrane filters (hollow fibre) or by cell immobilization onto an interaction surface of two immiscible liquids (Kourkoutas *et al*., 2004; Verbelen *et al*., 2006). The method based on the entrapment of cells in microcapsule or

encapsulations, consists firstly in entrapping the cells in a spherical gel and posterior coating with a polymer such as polyethyleneimine. Then, the gel is dissolved but the cells are left in suspension, contained behind the polymer barrier. The microporous membranes filters are normally made of polymers, *e.g*., polyvinylchloride or polypropylene (Margaritis and Kilonzo, 2005). The containment of the cells behind a barrier allows very high cell concentrations. For this reason, the membranes used should be freely permeable to nutrients and products released during the fermentation (Strehaiano *et al*., 2006), as well as mechanically resistant. This method of immobilization is normally used when a cell free product is needed. The main disadvantages are related to mass transfer limitations and the possibility of membrane fouling caused by the cell growth (Gryta, 2002).

Cell aggregation or flocculation can occur naturally or by using artificial flocculating agents. It is a complex process connected with the expression of flocculation genes such as FLO1, FLO5, FLO8 and FLO11 (Verstrepen *et al*., 2003). Yeast flocculation is an attractive method because of its simplicity and low costs (Verbelen *et al*., 2006). The flocculation depends on various parameters such as pH, nutrients, dissolved oxygen, medium composition and fermentation conditions (temperature and agitation) as well as the age of the cell (Jin and Speers, 1998; Verstrepen *et al*., 2003). An important issue for the success of this system is the selection of a proper yeast strain and fermentation system. In food industry, the main applications of the flocculation are the alcohol production, some kind of beers and sparkling wines (secondary fermentation). The flocculation is very important for the brewing industry as it is an effective, environmentally friendly, easy and without costs method to separate the yeast cells from the green beer at the end of the fermentation (Verstrepen *et al*., 2003). The flocculation of the yeast is a very important characteristic also in the traditional making of sparkling wines (Torresi *et al*., 2011).

2.2.1 Types of supports

It is of the highest importance the selection of the immobilization support for further implementation in the food industry. The support must be easily accepted by the consumer and its selection depends on the process in which it will be applied as well as the process conditions. The support can be used in their natural form or submitted to some treatment to modify the surface of the support in contact with the biomass (Genisheva *et al*., 2011). In the Figure 2.2 is presented a classification of currently used supports.

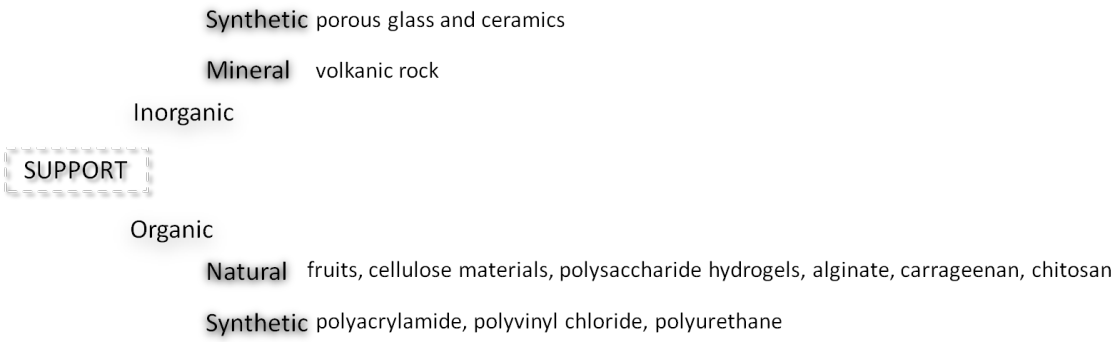

Figure 2.2. Classification of supports used for cell immobilization.

Several works have been published with inorganic supports like *kissiris* and *γ*-alumina. Inorganic supports are thought to be more attractive than organic supports due to their low cost, abundance in nature, reusability and are environmentally friendly. Studies with *kissiris* and *γ*-alumina demonstrated increased fermentation rates and ethanol productivity at ambient and low temperatures (Bakoyianis *et al*., 1997). Even though the wines produced with an inorganic support had improved aroma, these supports turns undesirable for winemaking because of the mineral residues left in the final product. A comparative study on *kissiris*, *γ*-alumina and calcium alginate as potential supports for cells immobilization, demonstrated that calcium alginate had the best results in winemaking, by representing a more stable environment for the entrapped yeast cells. At the same time, it was the most expensive and time consuming material. The cheapest and the more abundant support mentioned above is the *kissiris*, followed by *γ*-alumina (Bakoyianis *et al*., 1997).

The organic support from natural sources has received higher attention for wine production. Parts of fruits are the most common support used for batch or continuous winemaking. Wines were produced using apple cuts (Kourkoutas *et al*., 2002a), quince (Kourkoutas *et al*., 2003), watermelon (Reddy *et al*., 2008), dry raisin berries (Tsakiris *et al*., 2006), grape skins (Mallouchos *et al*., 2002), pear (Mallios *et al*., 2004) and others. Even though the aforementioned fruits, apple, quince and dry raisin berries are appropriate for winemaking, their cultivation, availability and cost are limited for industrialization (Reddy *et al*., 2008). Lately, whole grains of wheat, corn and barley were used for cell immobilization (Kandylis *et al*., 2010, 2012a and 2012b). These natural products are interesting in terms of compatibility with the final product and it is expected that they will not interfere or will bring positively changes to it. Moreover the natural origin of these supports induces an easier acceptance by the consumer.

Another natural material widely used as support for immobilization is the delignified cellulosic material. The cellulosic material is alcohol resistant giving high operational stability

in alcoholic fermentation. Moreover, it is a solid with low market value that does not release any contaminants into the final product (Iconomou *et al*., 1995).

Polysaccharides are originated from renewable sources such as algae, plants and selected microbial strains, and are normally considered to be more economically profitable over the synthetic polymers (Coviello *et al*., 2007). Polysaccharides are a class of polymers with a complex structure bringing a large variety of composition and properties. One of the most widely known and used polysaccharide is the alginate; it can be extracted from marine brown algae or produced by bacteria. It is considered to be one of the best matrices to entrap whole microbial cells, because gelification is carried out under very mild conditions. Moreover a large amount of cells can be immobilized, the substrates and products can easily cross the support and cell leakage is small (Spetolli *et al*., 1982). To prevent the cell leakage from the beads new approaches were used such as the technique of coating alginate beads (Crapisi *et al.*, 1992) or using beads with double-layers (Yokotsuka *et al*., 1997). Another well-known polysaccharide is carrageenan. It is obtained by extraction of certain species of red seaweeds. There are different types of carrageenan depending on the degree of sulfation (normally between 15 % and 40 %), identified traditionally by a Greek prefix (Coviello *et al*., 2007). From the three commercially most important carrageenans, ι-(mono-sulfate), κ-(di-sulfate), and λ-carrageenan (three-sulfate), κ-carrageenan is the one already used as support material for wine production (Crapisi *et al*., 1987).

When choosing a proper support for cell immobilization some aspects must be considered, like price of the material, ease of regeneration, cell load, type of immobilization, stability, rigidity, sterilization, possibility to use in different reactor designs and approval for food use (Virkajärvi and Linko, 1999).

2.2.2 Advantages and disadvantages of immobilized cell systems

The main advantages of the cell immobilized fermentations are:

- improved productivity, high volumetric reaction rates and high specific product yields
- regeneration of the biocatalyst activity of the immobilized cell systems after storage
- reutilization of the immobilized cell systems
- adaptation to continuous processes
- simplified systems for removing microbial cells, easier downstream processing
- greater tolerance of the cells to inhibitory substances
- biological stability at prolonged operation times, long term stabilization of cell activity
- smaller scale fermentation facilities (reduced capital and running costs)
- better control and conduction of the fermentation processes

When using immobilized cell systems some disadvantages must also be considered such as mechanical stability of the matrix used to immobilize microbial cells or loss of activity on prolonged operation.

2.3 Wine production with immobilized cells

Immobilization technology is used in various fermentation processes. Immobilized cells were used for bioethanol production (Rakin *et al*., 2009), cider production (Scott and O´Reilly, 1996), vinegar production (Ory *et al*., 2004) and brewing (Brányik *et al*., 2004a) as well as for winemaking (Table 2.1). Not many works are published for alcoholic fermentation of grape must and little are for malolactic fermentation of wine.

In our days, the induction of alcoholic fermentation and malolactic fermentation is done with starter cultures of cells, *i.e*. pure culture of cells isolated and developed for conducting wine fermentations. Most fermenters used in the winemaking industry are of a batch type, *i.e.* separate lots (batches) and are individually fermented till conclusion of the process (Jackson, 2008). Some industries adopted continuous methods, because of its advantages in controlling the yeast population and activity, keeping them in their maximum (Ribéreau-Gayon *et al*., 2006). The environmental conditions of continuous fermentations are favourable for the yeast growth, thus the biomass concentration is approximately two times greater than traditional winemaking (Ribéreau-Gayon *et al*., 2006). One of the most important characteristic of the continuous process is the high volumetric productivity (Verbelen *et al*., 2006) but, despite of its potential advantages, it is only rentable when working all year-round (Jackson, 2008; Ribéreau-Gayon *et al*., 2006). Immobilized cell systems emerged as a technique that provides also large amounts of cells but is more economic than the free cells continuous winemaking (Jackson 2008). Immobilized cell systems (ICS) give the possibility to produce new styles of beverages, with low alcohol content and very aromatic, and facilitate the conduction of fermentations where convenient removal of yeast cells is desired like the champagne method (Diviès and Cachon, 2005).

For implementation of the immobilized systems in industrial wine production, it is important to identify a suitable support for cell immobilization that is of food-grade purity, abundant, of low cost and which may contribute to an overall improvement, or not to interfere negatively, in the sensory characteristics of the final product (Kourkoutas *et al*., 2003).

During the process of winemaking, immobilized cell systems can be used in the alcoholic, malo-alcoholic and malolactic fermentations as well as for production of sparkling wines (Table 2.1).

Table 2.1. Immobilization type, supports, mode of operation, microorganisms and bioreactor operation conditions used in winemaking

Fermentation type	Microorganism	Support	Immobilization type	Operation mode	Bioreactor/conditions	Reference
primary, alcoholic	*S. cerevisiae*	Ca-alginate, single double layer	entrapment	batch	10 °C to 40 °C	Yajima and Yokotsuka, 2001
primary, alcoholic	*S. cerevisiae* (Montrachet 522)	κ-carrageenan	entrapment	continuous	tapered packed bed column, 13 °C	Uematsu *et al*., 1988
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	delignified spent grains	thermal dried, attachment	batch	15 °C	Tsaousi *et al*., 2010
primary, alcoholic	*S. cerevisiae Uvaferme299*	raisins	attachment	batch	packed bed reactor 6 °C to 30 °C	Tsakiris *et al*., 2004
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	Freeze-dried gluten pellets	entrapment	batch continuous	multi-stage fixed bed reactor, packed bed, 5 °C to 30 °C	Sipsas *et al*., 2009
primary, alcoholic	*S. cerevisiae*	*kissiris*, *γ*-alumina, Ca-alginate	attachment, entrapment	batch, continuous	two linked glass tower reactors 7 °C to 20 °C	Bakoyianis *et al*., 1997
primary, alcoholic	*S. cerevisiae CFTRI (101)*	watermelon	attachment	batch	15 °C to 35 °C	Reddy *et al*., 2008
primary, alcoholic	*S. cerevisiae CFTRI (101)*	guava	attachment	batch	15 °C to 35 °C	Reddy *et al*., 2006
primary, alcoholic	*S. cerevisiae*	orange peel	attachment	batch	15 °C to 30 °C	Plessas *et al*., 2007
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	brewer´s spent grains	attachment	batch	10 °C to 25 °C	Mallouchos *et al*., 2007
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	delignified cellulosic material, gluten pellets	attachment	batch	10 °C to 20 °C	Mallouchos *et al*., 2003
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	grape skin	attachment	batch	from 10 °C to 25 °C	Mallouchos *et al*., 2002

Table 2.1. Immobilization type, supports, mode of operation, microorganisms and bioreactor operation conditions used in winemaking(cont.)

Fermentation type	Microorganism	Support	Immobilization type	Operation mode	Bioreactor/conditions	Reference
primary, alcoholic	*S. cerevisiae*	γ-alumina	attachment	batch, continuous	multi-stage fixed bed reactor, packed bed, 7 °C to 27 °C	Loucatos *et al*., 2000
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	quince	attachment	batch, continuous	packed bed reactor, from 5 to 30°C	Kourkutas *et al*., 2002b
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	pear	attachment	batch, continuous	packed bed reactor, from 5 °C to 30 °C	Mallios *et al*., 2004
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	apple cuts	attachment	batch, continuous	packed bed reactor, from 5 °C to 30 °C	Kourkutas *et al*., 2002a
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	delignified cellulosic material	attachment	batch	packed bed reactor, from 0 °C to 30 °C	Bardi and Koutinas 1994
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	corn starch gel	entrapment	batch	from 2 °C to 30 °C	Kandylis *et al.*, 2008
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	wheat grains	attachment	batch	from 5 °C to 30 °C	Kandylis *et al.*, 2010
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	corn grains	attachment	batch	5 °C to 30 °C	Kandylis *et al.*, 2012a
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	barley grains	attachment	batch	5 °C to 30 °C	Kandylis et al., 2012b
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	gluten pellets	attachment	batch	packed bed reactor 5 °C to 30 °C	Iconomopoulou *et al*., 2002
primary, alcoholic	*S. cerevisiae* (AXAZ-1)	delignified cellulosic material	attachment	batch, continuous	packed bed reactor, 25 °C	Iconomou *et al*.,1995
primary, alcoholic, malo-alcoholic	*Schiz. pombe* *S. cerevisiae*	Ca-alginate	entrapment	continuous	multi-stage packed-bed, 25°C	Ogbonna *et al*., 1989

Table 2.1. Immobilization type, supports, mode of operation, microrganisms and bioreactor operation conditions used in winemaking(cont.)

Fermentation type	Microorganism	Support	Immobilization type	Operation mode	Bioreactor/conditions	Reference
primary, malo-alcoholic	*Schiz. pombe*	Ca-alginate, double layer	entrapment	batch	20 °C	Silva *et al*., 2003
secondary, bottle	*S. cerevisiae*	Ca-alginate, double layer	entrapment	batch	15 °C or 25 °C	Yokotsuka *et al*., 1997
secondary, bottle	*S. cerevisiae*	Ca-alginate	entrapment	batch	11 °C to 14°C	Fumi et al., 1988
secondary, malolactic	*O. oeni*	positively charge fibrous sponge	attachment	batch	28 °C	Maicas *et al*., 2001
secondary, malolactic	*O. oeni*	corn cobs grape skins grape stems	attachment	batch	25 °C	Genisheva *et al*., 2013
secondary, malolactic	*L. oenos*	Ca-alginate	entrapment	batch	Stirred, 20 °C	Spetoli *et al*., 1982
secondary, malolactic	*Lactobacillus*	κ-carrageenan	entrapment	continuous	column, from 7 °C to 40°C	Crapisi et al., 1987
secondary, malolactic	*O. oeni* (ATCC 23279)	delignified cellulosic material	attachment	batch	glass cylinder, 27 °C	Agouridis *et al*., 2008
secondary, malolactic	*L. casei* (ATCC 393)	delignified cellulosic material	attachment	batch	glass cylinder, 20 °C	Agouridis *et al*., 2005
secondary, malolactic	*L. oenos PSU-1*	κ-carrageenan	entrapment	batch	25 °C	McCord and Ryu 1985
secondary, malolactic	*L. casei*	pectate gel, modified chitosan beads	entrapment, attachment	batch	shaking, 20 °C, 25 °C and 36 °C	Kosseva *et al.*, 1998
secondary, malolactic	*I.orientalis* (KMBL 5774)	mixture of oak charcoal and sodium alginate	entrapment	batch	shaking 30 °C	Hong *et al.*, 2010

2.3.1 Primary alcoholic fermentation

2.3.1.1 Alcoholic fermentation with immobilized cells

Alcoholic fermentation is the area where the immobilized cell systems are mainly used in winemaking. According to Table 2.1 the most used microorganism for the alcoholic fermentation of wines is the *Saccharomyces cerevisiae*. Depending on the country where the study was made and according to the tendencies of protecting the typical and restricted characteristics of the local wines, most of the times locally isolated strains of *S. cerevisiae* are used.

Bakoyianis *et al*. (1997) used three different supports for the immobilization of an alcohol-resistant strain of *S. cerevisiae*. Yeast cells were immobilized on *kissiris* (volcanic rock), *γ*-alumina and calcium alginate and further applied for wine production at different temperatures. From the three solid supported biocatalysts, calcium alginate presented the highest fermentation rates and ethanol productivity at low temperatures. *Kissiris* is considered to be a good option for immobilization support as it is abundant in nature, environmentally friendly and can be easily regenerated. The use of γ-alumina in winemaking implies an additional step in the process, *i.e.* the removal of the aluminium from the produced wine (Loukatos *et al.*, 2000).

Natural supports of food-grade purity like delignified cellulosic material (Bardi and Koutinas, 1994) and gluten pellets (Bardi *et al*., 1996) were used successfully as immobilization supports for winemaking at ambient and low temperatures (from 0 °C to 30 °C) . This ICS caused about a three-fold increase of the fermentation rate when compared with free cells; moreover the ethanol productivity and daily wine production were higher. Sipsas *et al.*, (2009) also used yeast cells immobilized on gluten pallets, which were subsequently freeze-dried. The system showed high operational stability, even after storage for 6 months at 4 °C and produced wines with an improved quality.

In order to find a suitable support for immobilization that corresponds to the prerequisites of food-grade purity together with consumer acceptance, researchers proposed pieces of fruits or whole grains. Yeast cells immobilized on orange peel showed to be a suitable biocatalyst for commercial applications (Plessas *et al*., 2007). This ICS was used for alcoholic fermentation at different temperatures resulting in high ethanol productivity and low fermentation times. Watermelon pieces were also used as immobilization support for winemaking at different temperatures (Reddy *et al*., 2008). This ICS improved the fermentation rates, the viability and vitality of the immobilized yeast cells. The produced wines were found to be with good taste and with improved quality. The main drawback of this system was the significant loss of

watermelon volume; however, after the seventh or eighth batch the watermelon pieces volume stayed constant. The studies carried out by Kourkoutas *et al.* (2001 and 2003) with apple cuts and quince cuts as support materials, also observed an important decrease of the immobilized support during the first batches. Nevertheless the immobilized cells were able to produce wines at low temperatures (0 °C, 5 °C and 10 °C) and kept their biocatalyst activity for of at least 7 months. Tsakiris *et al*. (2004) used yeast cells immobilized on raisin for the production of red wine at different temperatures. Kandylis *et al*., (2010, 2012a and 2012b) used whole grains of wheat, corn and barley as support materials for yeast immobilization. The resulting wines had improved aromatic profiles when compared to fermentations with free cells.

2.3.1.2 Malo-alcoholic fermentation with immobilized cells

The fission yeast *Schizosaccharomyces pombe* efficiently degrades high concentrations of L-malic acid by means of malo-alcoholic fermentation. However, the use of *Schiz. pombe* in vinification may be unsuitable as this yeast can produce undesirable off-flavours in the wines Yokotsuka *et al.*, 1993. During the malo-alcoholic fermentation malic acid is directly transformed into ethanol. Some studies using *Schiz. pombe* in immobilized cell systems were made. Malo-alcoholic fermentation with immobilized *Schiz. pombe* cells, even though is not a perfect alternative to the malolactic fermentation, can improve the acid harmony of wines with high acidity (Maygar and Panyik, 1989).

Schiz. pombe cells normally are immobilized in Ca-alginate beads (Ciani, 1995; Maygar and Panyik, 1989) or fibres (Yokotsuka *et al.* 1993). This ICS can be used for deacidification of grape must before alcoholic fermentation (Silva *et al*., 2003; Yokotsuka *et al.* 1993), or for degradation of malic acid in wines (Ciani *et al*., 1995; Maygar and Panyik, 1989). In some cases the *Schiz. pombe* immobilized cells were still active after 20 months of storage; moreover, the alginate beads with entrapped cells could be recycled up to five times without cell leakage (Silva *et al*., 2003). Sometimes the resulting wines had small amounts of sediments and little distinct off-flavour (Yokotsuka *et al.* 1993). However, most of the authors concluded that wines obtained by this method had better organoleptic quality than the wines without previous deacidification (Silva *et al*., 2003), and no off-flavour or off-taste were detected (Ciani, 1995; Maygar and Panyik, 1989).

2.3.2 Secondary wine fermentation

2.3.2.1 Malolactic fermentation with immobilized cells

Normally the supports used for conducting malolactic fermentation (MLF) in wines are from organic origin. The bacterial cells used in immobilized cell systems (ICS) for MLF are

Lactobacillus or *Oenococcus oeni* (formerly known as *Leuconostoc oenos* and reclassified by Dicks *et al*., 1995).

Leuconostoc oenos (today know as *O. oeni*) cells were immobilized on calcium alginate gels to be used for conducting MLF in red wines (Spetolli *et al*., 1982). Even though this ICS showed high reaction yields and small number of cells leaked from the gel (0.1 %), the operational activity of the system declined gradually with time (after 17 d).

Crapisi *et al.* (1987) used *Lactobacillus* cells immobilized on *κ*-carrageenan gel for controlling and conducting MLF. The conversion ratio of malic acid was 53.9 %, and the sensory properties of the wine stayed unchanged.

Calcium pectate gel and chemically modified chitosan beads were used as supports for immobilization of *Lactobacillus casei* (Kosseva *et al.*, 1998). Repeated batch fermentations were carried out with different wine samples and at different temperatures (35 ^{o}C, 25 ^{o}C, 20 ^{o}C). The temperature was found to be the main factor affecting the rates of the MLF. The best fermentation rates were recorded for assays conducted at 25 ^{o}C, where malic acid decreased 30 % within 1 h. The degradation rate of malic acid using immobilized cells was twice as high as that obtained with free cells. These ICS were found to be with potential for industrial application as they showed long term operational stability; calcium gel beads were stable for 6 months and chitosan beads for 2 months. Another study from the same authors (Kosseva and Keneddy, 2004) demonstrated that encapsulated *L. casei* in a pectate gel also increased the fermentation rates and, moreover, makes the fermentation to take place at high ethanol concentrations (12 % vol. to 13 % vol.).

However, the encapsulation method has mass transfer limitation of nutrients that leads to inactivation, or even death, of the cells in the centre. Therefore, a new immobilization support was proposed: a fibrous sponge which is cellulose based (Maicas *et al*., 2001). The surface of the sponge can be modified and ionized. Maicas *et al*. (2001) showed that the positively charged sponge immobilized the highest amounts of *O. oeni* cells and used this ICS for MLF in red wines. Although the results were better than assays performed with free cells, a decrease of the activity of the immobilized cells was detected after 4 to 6 repeated batches. The main reason was considered to be the diminished viability of cells after long exposure to ethanol.

Agouridis *et al.* (2005) also used a cellulosic material for the immobilization of *L. casei* and conducted MLF at 27 °C. Once again with the repeated batch fermentation (more than 1 month) the malolactic activity of the immobilized cells decreased. Nevertheless, the authors concluded that the delignified cellulosic material (DCM) is a promising support for MLF, but more research is required for improving some parameters. In another study from the same authors the DCM was used for the immobilization of *O. oeni*, strain that is highly resistant to ethanol. In

this study the authors demonstrated a good operational stability of the ICS during all 11 repeated batch fermentations. The malic acid degradation could be maintained stable within an average value of 54.0 %.

2.3.2.2 Sparkling wines produced with immobilized cells

In the traditional production of sparkling wines, lees removal is a very laborious and time-consuming process and the use of immobilized yeasts has been investigated in order to diminish and simplify the riddling and disgorging procedures. Among the available immobilization techniques, encapsulation in polysaccharide gels such as alginate is the most widely used. Immobilized yeasts are commonly used in sparkling wine production in experimental settings; however their application in oenological practices is still uncommon (Toressi *et al*., 2011).

Immobilized *S. cerevisiae* cells on calcium alginate were used for sparkling wine production (Fumi *et al*., 1988). Cells were released from the beads but with little influence on the clarity of the wine, according to the tasters. However, there were not found differences between the wines obtained with immobilized cells and wines obtained by the traditional method in terms of the main components: ethanol, organic acids and higher alcohols.

For preventing cellular leakage from the beads, Crapisi *et al*. (1992) used coated alginate beads and were able to obtain a biologically stable sparkling wine. Sparkling wines produced with free and immobilized cells were not found different in terms of aromatic compounds.

Yokotsuka *et al*. (1997) used *S. cerevisiae* cells immobilized in double-layer gel beads or strands for the bottle-fermentation. The beads were easily inserted in the bottle and simply removed in ice plugs during disgorging. The produced sparkling wine was clear and similar in taste and *bouquet* to that made using free yeast. Moreover, with the increase of the amount of beads the calcium content in the sparkling wine also increased.

2.4 Reactors used with immobilized cells

Reactors operating with immobilized cells have higher productivity and operational stability, as well as easier downstream processing. Another attractive advantage of the immobilized cell bioreactors, compared to the existing free cell fermenters, is the faster fermentation time. Because of this and other benefits the immobilized cell bioreactors have been applied in many industrial processes, including beverage production. Choosing the proper reactor for use with immobilized cell systems depends on the type of immobilization, the type of the used support, mass transfer requirements and conditions of the process. For example, it is of a big importance the resistance of the immobilized cell system to the shear forces as well as the size of the

support. According to the type of immobilization procedure and support used, an appropriated reactor must be designed.

2.4.1 Continuous reactors with immobilized cells

The biological reactors mostly used in the industries can have different operation modes: batch or continuous. Batch reactor is "closed reactor", *i.e.* once inoculated, no further inputs of nutrients or outputs of products occur. In this type of reactors the velocity of cell growth tends to zero. It is one of the most used reactors in a big variety of industrial processes. The batch reactor can be stirred or not stirred.

The fed-batch reactor is a variance intermediate between batch and continuous reactors. It is an "open reactor" like the continuous, but operates on an unsteady-state basis like the batch reactor. The main characteristic of the fed-batch system is to control the inflow of the growth limiting nutrients, leading to high cell densities in the bioreactor. The controlled addition of nutrients, affects the growth rate of the cells and helps to avoid formation of site metabolites.

The continuous reactor is an "open reactor" where there is a constant inflow of nutrients and outflow of product. The main characteristic of the continuous reactor is the possibility of reaching a dynamic equilibrium, *i.e*. the system operates on steady-state basis. Continuous reactors are used widely in the food, pharmaceutical and chemical industries. For continuous production the most used reactors are the multiphase reactors, including packed bed reactor, fluidized bed reactor, bubble column and air-lift reactor (Verbelen *et al*., 2006). The multiphase rectors include three phases: solid (the support), liquid (the medium) and gas (air, or other).

2.4.1.1 Packed bed reactor

Packed bed or also known as fixed bed reactor is extensively used in the chemical, petrochemical and biotechnology industries (Larachi *et al*., 1997). In this reactor type the immobilized cells are packed inside the reactor and a co-current of gas and fermentation media is passed upflow (flooded bed reactor) or downflow (trickle-bed reactor). The efficient performance of these reactors depends on gas-liquid mass transfer, which is strongly influenced by the gas-liquid interfacial area (Larachi *et al*., 1997). Despite its simplicity, during the operation of a packed bed reactor the following drawbacks can take place: channelling, fouling, mass transfer limitations, difficulties in CO_2-evacuation and compression of some support materials (Verbelen *et al*., 2006).

2.4.1.2 Fluidized bed reactor

In fluidized bed reactor the cells are attached and grow onto an inert support and the fermentation liquid is fed as at an upstream flow above the "minimum fluidization velocity" that

guarantees the fluidization of the support particles. In this system there is a vigorous mixing of gas, liquids and solids by the upstream flow. When using this reactor, it is important to take in consideration the specific weight of the support used for cell immobilization, as too light support will result in wash-out (Verbelen *et al*., 2006). The main advantages of this reactor type are high biomass concentration and surface area, good transfer of nutrients, high substrate utilization rates, low pressure drop across the bed and good process control (Nicolella *et al.*, 1997; Rovatti *et al*., 1995). Fluidized bed reactors are also generally used in wastewater treatment.

2.4.1.3 Air-lift reactor

In air-lift reactor the mixing of the liquid is provided by the injection of gas. There are two types of air-lift reactors – "internal" and "external" loop. The air-lift consists of two tubes linked together on the top and on the bottom. In one of the tubes (riser), the air is injected at the bottom, while normally in the other tube no air is injected (downcomer). The loop liquid circulation is caused by the density differences between the riser and the downcomer. The ideal cell support for air-lift reactor should have a low enough terminal settling velocity to be suspended by the upflowing gas and liquid streams. When comparing air-lift reactor to bubble column reactor or stirred tank reactor shear stress is mild and constant throughout the reactor. These types of reactors are economical as the aeration requests low-energy input, can be easily scaled up and used commercially (Couvert *et al.*, 1999).

2.4.1.4 Bubble column reactor

In a bubble column reactor, gas is injected in the bottom of the reactor through a gas distributor. Kulkarni *et al*. (2008) compared the motion of the bubbles to that of a swarm. Moreover the gas phase moves homogeneously or heterogeneously in a continuous liquid phase. The homogeneous gas regime occurs when the superficial gas velocity is lower than 5 cm/s. The size and the concentration of the bubbles in this type of regime are uniform. The heterogeneous gas regime occurs at high superficial gas velocity. The characteristic of this type of regime is the presence of radial hold-up profiles; originating intense liquid circulation (Joshi, 2001). Bubble column reactors are commonly used in the chemical industry as they are simple in construction and operation, have high mass and heat transfer rates, without any moving parts, are compact and have low operation and maintenance costs (Kantarci *et al.*, 2005; Tabib *et al*., 2008). Nevertheless, this type of reactor presents some disadvantages such as local flow, turbulence and gas hold up and complex hydrodynamics.

All these reactors were used at different production processes. The most used reactors with immobilized cells, for vinegar production, in laboratory conditions, are the packed bed reactor,

the batch reactor and the fluidized bed reactor (Ory *et al.*, 2004). The main reactor types used for continuous beer production with immobilized cells are the packed bed reactor, the air-lift reactor and the fluidized bed reactor (Brányik *et al*., 2004b; Verbelen *et al*., 2006). There are limited reports for continuous production of cider with immobilized supports. Herrero *et al*. (2001) produced cider continuously in Erlenmeyer flasks with cells entrapped in alginate beads. Nedovic *et al*. (2000) reported the successful used of continuous packed bed reactor in cider production.

2.4.2 Immobilized cell reactors used in winemaking.

Table 2.1 presents the applications of ICS and biological reactors in winemaking in the last 25 years. As it can be seen, a larger amount of works is made with winemaking in batch mode than in continuous mode. Moreover, most of the works are about alcoholic fermentations and less are about malolactic fermentation. It can be seen that for continuous production of wine the most used reactor type is the packed bed reactor. In the subsequent paragraphs some examples of batch and continuous winemaking are described.

Sipsas *et al*. (2009) used a multi-stage fixed bed tower reactor (MFBT) for winemaking in batch and in continuous. The MFBT operated at low temperatures (5 °C) and showed significant operational stability. Moreover the MFBT resulted in higher alcohol productivity of wines compared with packed bed reactor (PB). Nevertheless, the analyzed volatile compounds of the produced wines in MFBT and in PB reactors did not show significant differences between wines.

Tsakiris *et al*. (2004) used a 1.5 L tower glass reactor for batch production of red wine with cells immobilized on black and golden raisins berries. The fermentations were carried out with 300 mL of grape must and 100 g of immobilized support, at temperatures between 6 °C and 30 °C. The fermentation times for the different temperatures were as follows: 35 h to 40 h at 30 °C; 4 d at 22 °C and; 8 d at 6 °C. The sensory evaluation of the produced wines showed that the tasters preferred wines produced with immobilized cells rather than wines produced with free cells.

Kourkoutas *et al*. (2002a and 2002b) carried out continuous and batch fermentations of wines in a glass tower reactor with a total volume of 2 L. The volume of the grape must used in the experiments was 720 mL and the immobilized support added (apple or quince cuts) was around 1.2 kg. The batch fermentations with cells immobilized on apple cuts resulted in wines with high ethanol concentrations. The operation stability of the continuous system was for 71 d at least. Wine productivities in continuous mode of operation were much higher than in the repeated batch fermentations.

Ueamatsu *et al.* (1988) found extremely difficult to operate and control the conventional cylindrical type packed bed reactor. As a result they modified the system and used a tapered (conical) packed bed reactor for continuous wine production. ICS had improved fermentation performance compared to free cell fermentations. The new design bioreactor gave satisfactory results as well as operational stability for 2 to 3 months.

Bakoyianis *et al*. (1997) used three different support materials (*kissiris*, *γ*-alumina and calcium alginate) for batch and continuous winemaking. For the batch fermentations a 500 mL glass tower reactor was used, with 300 mL of grape must and the weight of each immobilized support was calculated so that the concentration of the immobilized cells is the same for all assays. The pilot plant for the continuous fermentations consisted of two glass reactors (each of 1.5 L total volume and 1.0 L liquid volume) linked together so that the outlet of the first reactor was the inlet of the second reactor. In the continuous fermentations the ethanol production was found to be 4 to 10 fold higher compared to batch fermentations. The three continuous systems were operated for 80 d without loose of operational activity.

In general there are less published studies about malolactic fermentation of wines conducted with immobilized cells. In 1998 the Bulgarian author Kosseva (Kosseva *et al*., 1998), published a work about conducting malolactic fermentation in Chardonnay wines with immobilized cells on two different materials (calcium pectate gel and chemically modified chitosan beads). Repeated batch fermentations were carried out at shake flask at different temperatures (Table 2.1). The degradation of malic acid was 30 % for 1 h, twice higher compared with free cell assays.

Agouridis *et al.* (2008) used for repeated batch malolactic fermentations a 1 L glass tower reactor. The average value of the malic acid degradation was 54 % and stayed stable for the 11 batch successive fermentations. Nevertheless, the average concentrations of produced lactic (0.98 g/L) and acetic acids (0.39 g/L) were low.

2.5 Continuous winemaking

Continuous fermentation process is a solution for reducing production costs as well as improving the process efficiency and ethanol yield (Vasconcelos *et al*., 2004). Continuous processes are preferred in most fields of industry because of the great economic advantages. The effort of implementing a continuous process is not always successful (Virkajärvi and Linko 1999), as there are some major issues linked to a process in continuous like keeping the system aseptic for long periods of time (at least for several months). If a system is contaminated and there is a need of stopping the process and making a new immobilization this increases the costs of the process and slows down the production. In winemaking, the continuous fermentation

system must be able to respond to another important issue that is the inhibitory effect of the formed products over the growth of the microorganisms (Virkajärvi and Linko 1999).

The main advantages of the continuous process are (Verbelen *et al.*, 2006):

- higher conversion rates;
- faster fermentation rates;
- improved product consistency;
- reduced product losses;
- environmental advantages.

Wine productivity in continuous winemaking with immobilized cells was found to be three to six folds higher than those obtained by natural fermentation (Iconomou *et al*., 1995). Nevertheless, continuous fermentations systems, common in other industries, are rarely used in the wine industry (Sipsas *et al*., 2009). Continuous fermentations with immobilized cells are very beneficial as it links high cell density with high flow rates that results in short residential times (Verbelen *et al*., 2006). The supports to be used for immobilization and further implementation for continuous winemaking should complete more prerequisites than low cost, abundance and food-grade purity. Besides the ones referred, it should also have the ability for long term storage, should have high resistance and stability and should not damage the quality of the final product (Genisheva *et al.*, 2012; Sipsas *et al.,* 2009).

Sipsas *et al*. (2009) produced wine in a continuous mode in a multi-stage fixed bed tower (MFBT) reactor, at different temperatures. Authors concluded that the continuous mode of operation and the fermentation temperatures affected the concentrations of ethyl acetate, amyl alcohols and methanol.

Reddy *et al*., (2008) used immobilized yeast cells on watermelon pieces in a continuous winemaking for 100 d at 20 °C, where the cells remained 90 % to 95 % viable.

Apple cuts (Kourkoutas *et al*., 2002a) and quince pieces (Kourkoutas *et al*., 2002b) were found to be suitable for wine production in continuous mode. Both ICS worked for 95 d and 46 d, respectively, without diminishing of the ethanol productivity. These systems were appropriate for working at low temperatures (5 °C) and the produced wines demonstrated improved quality compared to other commercial wines and distinctive flavour profiles, even though an increase of the total acidity was observed.

Continuous MLF was also studied by Crapisi *et al*. (1987) who used *Lactobacillus* cells immobilized on k-carrageenan gel. The ICS functioned for 46 d at temperatures between 7 °C and 40 °C.

Delignified cellulosic material was used as a support for cell immobilization and further applied in continuous process of winemaking (Bardi and Koutinas 1994). Wine productivity was six fold higher than in a traditional process. The ICS had an operational stability for 2 months (Iconomou *et al*., 1995).

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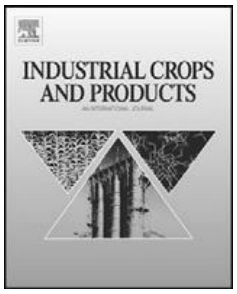
3. Evaluation of supports for yeast cell immobilization

Three winemaking residues (grape seeds, skins and stems), and corn cobs were evaluated as support material for immobilization of *Saccharomyces cerevisiae*. The main objective of this study was to find an abundant and low cost material suitable for the yeast cells immobilization and able to be used in wine production by immobilized yeast cells. The four natural materials were used as support in two different forms: untreated, and treated by a sequence of acid and basic reactions. Untreated grape skin and corn cobs provided the highest cell immobilization results, as well as the maximum ethanol production yield. It was also found that the support materials released nutrients to the medium, which favoured the yeast development and the ethanol production. Static fermentation with cells immobilized on grape skins or corn cobs appear to be an interesting alternative for use in winemaking. The use of grape skins, particularly, which is a by-product of the wine elaboration, could be of larger interest to obtain an integrated wine production process with by-product reuse.



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3.1 Introduction

Increased interest has been observed in the last years on the use of fermentation systems with immobilized cells, due to the several advantages that these systems present when compared to the conventional free cell fermentations. Such advantages include a higher cell concentration in the fermentation medium and consequently, an improvement in the process efficiency and productivity. Moreover, immobilized cell systems make it possible for cells to be easily recovered for later use in repeated batch operations, for example (Cohen, 2001; Genisheva *et al*., 2012; and 2013). Therefore, immobilized cell systems have been used in different fermentation processes to obtain a number of products, such as organic acids, edulcorants, oligosaccharides, beer, among others (Dragone *et al*., 2007; Meleigy and Khalaf, 2009; Mussatto *et al*., 2009; Silva *et al*., 2007).

Considerable attention has also been given to the cell immobilization of yeasts in winemaking. Some authors consider that immobilized cell systems offer many prospects for oenology, such as improved performance of alcoholic and malolactic fermentation, adaptation to continuous processes, and simplified systems for removing and reusing microbial cells in batch processes (Mallouchos *et al*., 2003). However, up till now the industrial application of this technology was not established due to some difficulties that have to be overcome. For example, as is well known, good performance of systems using immobilized cells mainly depends on the right selection of the immobilization support. For application in food and beverages industries, particularly, the support materials should be of food-grade purity, suitable for use under low-temperature fermentation and for long-term storage when necessary, and should contribute positively to the characteristics of the final product (Kourkoutas *et al*., 2006; Sipsas *et al*., 2009). As a whole, the supports to be used in immobilization belong to two major groups: natural organic and inorganic. Among these groups, several materials have been proposed for use in winemaking, including sodium alginate, Ca-alginate, *kissiris*, *γ*-alumina, gluten pellets, DEAE-cellulose, delignified cellulosic materials, fruit pieces, and dried raisin berries (Sipsas *et al*., 2009). Some authors consider inorganic supports more advantageous than the organic materials; nevertheless such supports have been found undesirable for winemaking because of the high concentrations of mineral residues in the final product (Kourkoutas *et al*., 2004).

Although several immobilization supports have already been proposed for alcoholic fermentation, only few of them find application at the industrial level, and therefore the search for new materials is of great interest. According to Kourkoutas *et al*. (2004), efforts should be concentrated on cheap, abundant, non-destructive and food-grade purity immobilization supports, which will improve quality and give a distinctive aroma profile and a fine taste to the

final product. Considering that the cost of the support material is a factor of significant influence on the final price of the product, interest has been paid to the use of agro-industrial residues for the cells immobilization. Corn cobs and grape pomace (composed by the grape skins, seeds and stems) are agro-industrial residues proceeding from the maize production and winemaking, respectively, available in large amounts in Portugal as well as in several other countries worldwide. Therefore, it is of interest to found alternatives for the reuse of these residues. The present Chapter evaluated the use of these residues for immobilization of the yeast *Saccharomyces cerevisiae*, aiming to find a material with suitable characteristics to promote the cells immobilization, as well as to obtain an efficient system for wine production by immobilized cells.

3.2 Materials and methods

3.2.1 Yeast strain and inoculum preparation

A commercial *Saccharomyces cerevisiae* strain (Lalvin QA23*,* Proenol*)* was used in the experiments. The inoculum was prepared by cultivation of the yeast in 500 mL Erlenmeyer flasks containing 200 mL of YPD medium with the following composition (g/L): yeast extract (10), peptone (20) and glucose (20). Cells were cultivated under static conditions, at 30 ^{o}C for 24 h, being subsequently recovered by centrifugation (*RCF*=7000, 20 min), washed with distilled water and re-suspended in the fermentation medium to obtain an initial concentration of 1 g/L (dry mass) at the beginning of fermentations.

3.2.2 Support materials for cell immobilization

Grape pomace (seeds, stems and skins), and corn cobs were used as support materials for the cells immobilization. The grape pomace was supplied by a local winemaking industry (*Divisão de Vitivinicultura – Direcção Regional de Agricultura e Pescas do Norte*), and the corn cobs were obtained from local farmers. Particles of seeds, stems and skins were separated from the grape pomace to be individually used in the experiments. The grape stems were cut in pieces of approximately 1 cm, while the grape skins were crushed in order to have an area close to 0.5 cm^2, and the grape seeds were used in their natural form. Corn cobs were ground and sieved, and only the particles with size between 0.45 mm and 2 mm were used as immobilization support.

All the support materials were used in the experiments in two different forms: untreated and treated. The untreated supports were only washed with distilled water and dried at 60 ºC until constant weight. Treated supports were prepared by mixing the materials with 3 % (v/v) HCl solution (15 mL/g), and maintaining at 60 °C for 2.5 h. The remaining solids were separated,

washed with distilled water until neutral pH and dried at 60 °C until constant weight. Subsequently, the acid-treated material was mixed with a 20 g/L NaOH solution (10 mL/g), at 120 min^{-1}, 30 °C for 24 h. Finally, the solid residue was separated from the liquid fraction, washed with distilled water until neutral pH, and dried at 60 °C until constant weight.

3.2.3 Fermentation medium and conditions

Fermentation experiments were carried out in 500 mL Erlenmeyer flasks containing 2 g of support material and 200 mL of culture medium composed by (g/L): glucose (120), yeast extract (4), $(NH_4)_2SO_4$ (1), KH_2PO_4 (1), $MgSO_4$ (5). The flasks were inoculated with 1 g/L of cells and their immobilization occurred *in situ* by natural adsorption through the direct contact with the support materials. The fermentations were performed at 30 ^oC for 24 h, under static conditions. For comparison, assays under the same conditions described above but without the support addition, were also performed. Samples were taken periodically for estimation of biomass concentration, glucose consumption and ethanol production.

To evaluate the nutritional effect of the support materials on the fermentation performance, the material was put in contact with the fermentation medium during 30 h, under static conditions. After this time, it was removed and the fermentation medium was inoculated with the yeast strain. Then, the ethanol production by free cells was performed under the same operational conditions described above. For comparison, fermentation runs with free cells were also carried out but without the previous stage of contact between the support and the fermentation medium.

The assays for evaluation of the influence of agitation on cells immobilization and ethanol production were performed under the same operational conditions described above, but in a rotary shaker at 150 min^{-1}.

3.2.4 Analytical methods

Glucose and ethanol concentrations were determined by High Performance Liquid Chromatography (HPLC) in a Jasco chromatograph equipped with a refractive index detector (Jasco 830-RI) and a Varian Metacarb 67H column (300 mm × 6.5 mm) operated at 60 ºC. A 50 mmol/L H_2SO_4 solution was used as eluent in a flow rate of 0.7 mL/min.

Immobilized cells concentration was determined at the fermentations' end, according to Brányik *et al*. (2004a) with slight modifications. At the end of the fermentations, the biocatalyst (carrier with immobilized cells) was separated from the liquid medium, dried at 60 ºC for 24 h and then, approximately 1 g was placed into an Erlenmeyer flask containing 20 mL of 30 g/L NaOH solution, and shaken at 30 ºC, 120 min^{-1}, during 24 h. After this time, the supernatant

was recovered and used for counting cells on a Neubauer chamber. Correlation between the number of cells and the corresponding cell concentration were made by using a calibration curve. The total removal of the immobilized cells from the support materials by means of the NaOH treatment was confirmed by scanning electron microscopy.

Free cells concentration in the fermentation medium was estimated by measuring the absorbance at 600 nm, which was correlated to a calibration curve (dry weight × optical density).

3.2.5 Scanning electron microscopy

Micrographs of the biocatalysts (after washing with deionized water and drying for 24 h at 60 ºC) were obtained by Scanning Electron Microscopy (SEM) using a Leica Cambridge S360 microscope. To be examined, the dried samples were fixed on a specimen holder with aluminium tape and then sputtered with gold in a sputter-coater under high vacuum condition. Each sample was examined at 700-fold magnification.

3.2.6 Fermentation parameters and statistical analysis

The ethanol yield ($Y_{P/S}$) was calculated by the ratio between ethanol produced and glucose consumed. The ethanol productivity (Q_P), was calculated by the ratio between the ethanol produced and the fermentation time.

All the fermentation experiments were conducted in duplicate. The results were analysed by analysis of variance (ANOVA) and where significant, difference in ANOVA ($p<0.05$) was detected by the Fisher's Least Significance Difference (LSD) multiple comparison test, which was applied to compare the differences among samples. Statgraphics Plus for Windows version 4.1 was the software used for data analysis.

3.3 Results and discussion

3.3.1 Influence of the support material and treatment stage on cells immobilization

Initially, the cells immobilization in the support materials as well as the effect of the treatment stage on the materials structure and cells adhesion was evaluated. Treatment of the materials by a sequence of acid and basic reactions previous they use as support promoted cleanness in the materials' structure, being observed mass losses for all of them. The lowest mass loss occurred for the grape seeds, being recovered 75 % of the original material mass after the treatment. For the other three evaluated materials (corn cobs, grape skins and grape stems), only 25 % of the original mass was recovered after treatment. The recovered mass yield is strongly related to the

original structure of the material. Grape seeds have a much harder structure than the particles of corn cobs, grape skins and grape stems, and probably, this structure hindered the acid and basic attack. When this sequence of treatment was applied for the brewer's spent grains, for example, a mass recovery yield of only 10 % was obtained. However, the authors concluded that such methodology was efficient to provide high immobilized cells load in this material support (Brányik *et al*., 2001).

The immobilized cell concentration on each untreated and treated support material at the end of the fermentation is shown in Table 3.1. It can be noted in this table that, for most of the cases, the cells were immobilized in larger amounts in untreated materials than in the treated ones. Among these, grape skins and corn cobs gave the highest immobilized cell concentration, which were not statistically different from each other at 95 % confidence level. However, these results were different (p<0.05) of those obtained for grape stems and seeds. Similar statistical differences were observed for the analysis of the treated supports, with the best results being also found for grape skins and corn cobs. It is worth mentioning that considerable values of immobilized cells were obtained in a short period (24 h), when using these two materials as support. Other authors obtained similar values of immobilized cells only after 75 h of fermentation (Brányik *et al.*, 2004b).

Table 3.1 Concentration of immobilized cells (X_{im}) ethanol concentration ($C_{et.}$), ethanol yield ($Y_{P/S}$) and productivity (Q_p) obtained during the fermentations using treated (T) or untreated (U) materials as support for the cells immobilization

Response	**Support material**							
	corn cobs		**grape stems**		**grape skins**		**grape seeds**	
	U	**T**	**U**	**T**	**U**	**T**	**U**	**T**
X_{im}/(mg/g)	22.20 ^{b,2}	19.95 ^{a,2}	4.08 ^{a,1}	2.83 ^{a,1}	25.10 ^{b,2}	9.28 ^{a,2}	1.68 ^{a,1}	2.38 ^{b,1}
$C_{et.}$/(g/L)	53.48 ^{a,1}	52.24 ^{a,1}	53.89 ^{a,1}	53.30 ^{a,1}	54.46 ^{a,1}	51.37 ^{a,1}	54.05 ^{a,1}	48.26 ^{a,1}
$Y_{P/S}$/(g/g)	0.51 ^{b,2}	0.40 ^{a,2}	0.44 ^{a,1}	0.38 ^{a,1}	0.49 ^{a,2}	0.50 ^{a,2}	0.51 ^{b,2}	0.39 ^{a,2}
Q_P/[g/(L h)]	3.35 ^{a,1}	3.27 ^{a,1}	3.37 ^{a,1}	3.33 ^{a,1}	3.41 ^{a,1}	3.21 ^{a,1}	3.38 ^{a,1}	3.02 ^{a,1}

a, b – for the same support material and to each response individually, values with the same letter mean no significant difference at 95 % confidence level, between U and T results.
1, 2 – to each response individually, considering only the treated or the untreated support materials, values with the same number mean no significant difference at 95 % confidence level among the results for the four supports.

The yeast cells were immobilized on the materials surface by adhesion, a natural phenomenon that is preferred in the beverage production over the use of potentially harmful inducers. Scanning electron microscopy of the grape skins and corn cobs (in the untreated and treated forms), before and after the cells adhesion (Figure 3.1), revealed that the immobilization did not occur in a homogeny form on the material structure, but it was more favoured in specific

regions, such as rough and porous structures. In fact, such structures allow microorganisms to attach more firmly to the materials surface than the smooth structures. This phenomenon has also been reported in other immobilization studies (Brányik *et al*., 2004b; Kosaric and Blaszczyk, 1990; Yu *et al*., 2010).

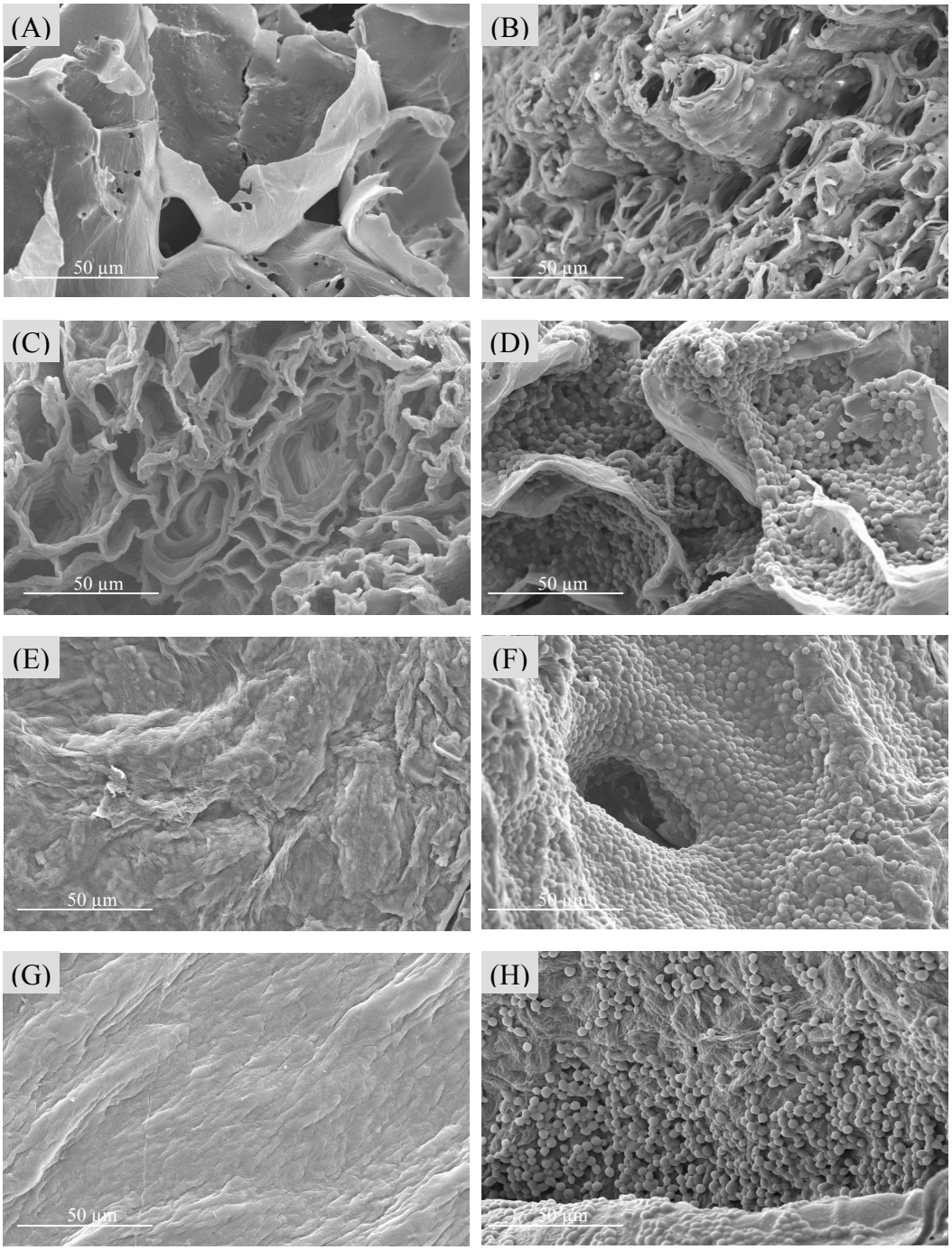

Figure 3.1. Micrographs by scanning electron microscopy (SEM) of the support materials used for cells immobilization. Untreated corn cobs before (A) and after (B) the cells immobilization; Treated corn cobs before (C) and after (D) the cells immobilization. Untreated grape skins before (E) and after (F) the cells immobilization; Treated grape skins before (G) and after (H) the cells immobilization. Magnification: 700-fold.

Grape skins and corn cobs gave higher immobilized cells concentration than the other two evaluated materials, both when used in the untreated or treated forms (Table 3.1). However, when observing the structure of these two materials, it is evident the differences between them (Figure 3.1). The untreated and treated corn cobs structures have many cavities (Figure 3.1 A and C), which provided a natural entrapment of the cells. On the other hand, grape skins have not many cavities (Figure 3.1 E and G) but their rough structure was probably the responsible for the elevated cells adhesion, mainly in the untreated form. In this case, due to the few amounts of cavities in the material surface and the large amount of immobilized cells, it was observed the formation of a biofilm with multiple layers of cells (Figure 3.1 F). Similar behaviour has been observed during the cells immobilization in treated brewer's spent grains (Brányik *et al.*, 2001). It is also important to observe in these figures that the grape skins structure was "cleaned" after the treatment, *i.e.*, most of its roughness appears to have been eliminated during this process (Figure 3.1 E and G). Such observation is in agreement with the lower immobilization results observed for treated grape skins when compared to the untreated material (Table 3.1), since the cells adhesion would have been hindered in this smoother surface.

In brief, grape skins and corn cobs were the most suitable support materials for immobilization of *S. cerevisiae* cells during fermentation. Treatment of these materials by a sequence of acid and basic reactions did not improve the cells adhesion to the supports surface but on the contrary, a lower concentration of immobilized cells was obtained, probably due to some cleaning effect of the chemicals on the materials surface that reduced their roughness hindering the cells adhesion as a consequence.

3.3.2 Influence of the support material and treatment stage on fermentation performance

The time course of fermentations for ethanol production by immobilized or free cells is shown in Figure 3.2. It is evident from Figure 3.2 A and B that the glucose consumption was faster in media containing immobilized cells than in the medium containing only free cells. In fact, not only the substrate consumption was faster but also the ethanol production (Figure 3.2 C and D) and the free biomass formation (Figure 3.2 E and F) was higher in the assays containing immobilized cells than in those using only free cells. For most of these assays (using untreated or treated supports) the maximum ethanol production occurred at 16 h fermentation, time in which the substrate has been almost totally exhausted from the media. For this same fermentation time, the glucose consumption, ethanol production and biomass formation in the free cells assays attained practically half of the values obtained for the immobilization

experiments using untreated or treated support materials. This fact suggests that immobilized cells improved the fermentation rates and efficiency of bioconversion.

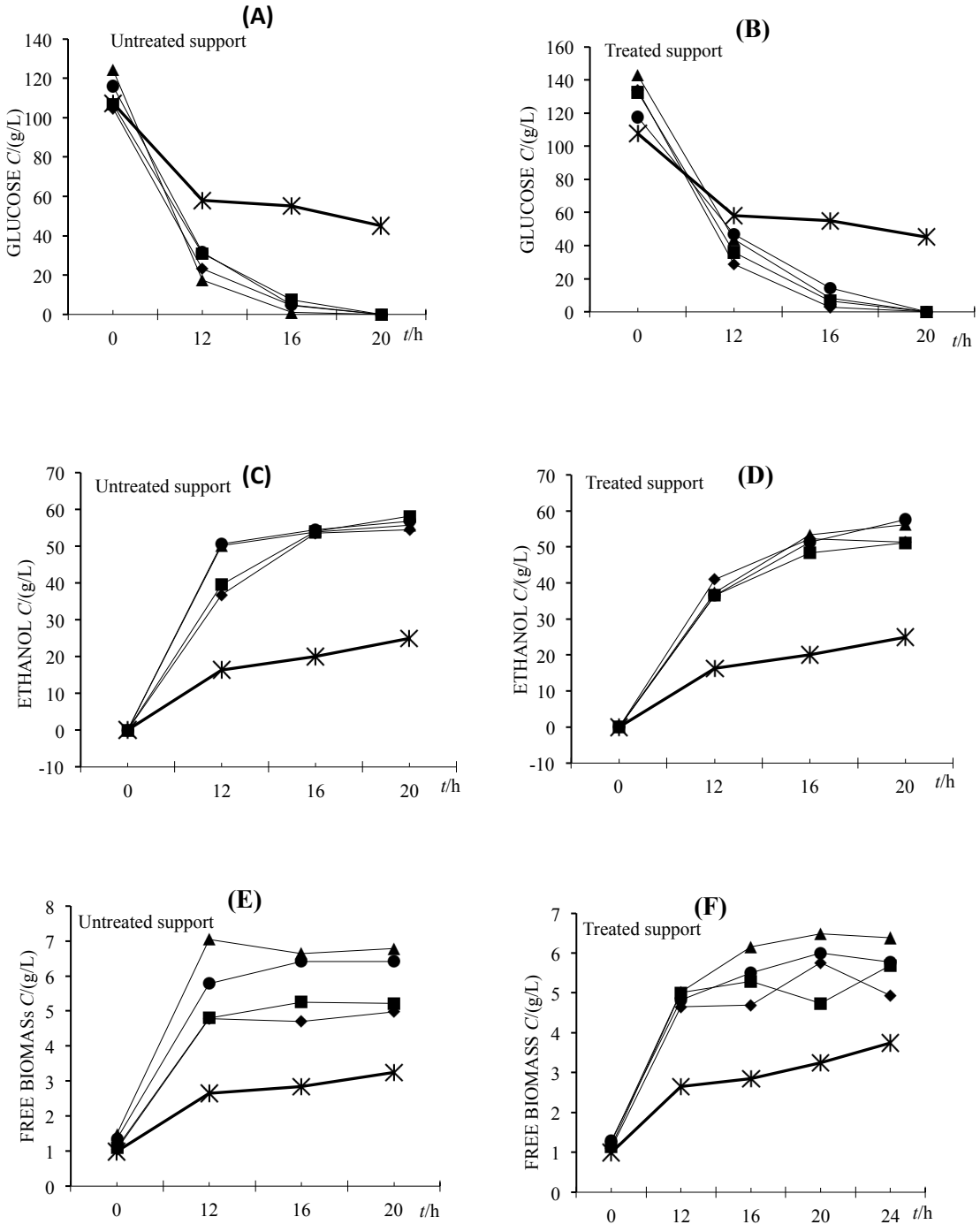

Figure 3.2. Time course of glucose consumption (A, B), ethanol production (C, D) and free cells formation (E, F) during the fermentation with cells immobilized on the different support materials (untreated and treated) and from the medium containing only free cells. Corn cobs (-◆-), grape stems (-▲-), grape skin (-●-), grape seeds (-■-), and free cells medium (-✳-). The standard deviation to each point represented in the curves is lesser than 10 %.

All the media containing immobilized cells gave similar maximum ethanol concentration (Figure 3.2 C and D) independently of the support material used, and if it was or not treated. This means that, even being obtained a significant higher amount of cells immobilized onto grape skins and corn cobs, the product formation in these media was not more elevated than in the experiments with cells immobilized in grape stems. Such fact is probably related to the amount of free biomass in these media. For all the immobilization assays, it was observed a high formation of free biomass (Figure 3.2 E and F), which would have contributed with the substrate consumption and product formation. Nevertheless, considering that the immobilized cells may be easily recovered from the medium and reused in subsequent fermentation stages, the elevated ethanol production achieved when using grape skins and corn cobs as immobilization material (which yielded the highest immobilized cells concentration) is a very advantageous aspect for future applications in continuous or repeated batch fermentation systems.

Although the similar ethanol production and glucose consumption for all the experiments with immobilized cells, the exact calculation of the ethanol produced per consumed substrate ($Y_{P/S}$) revealed a significant difference ($p<0.05$) among the performance of the fermentation using cells immobilized on grape stems and the assays using the other 3 support materials, both in the treated and untreated forms (Table 3.1), with grape stems assays giving the lowest ethanol yield ($Y_{P/S}$) values. The use of treated supports did not favour the ethanol production for any of the evaluated cases but, on the contrary, the ethanol production, yield and productivity were not different when using treated or untreated grape skins and grape stems, and the $Y_{P/S}$ value was worst when using treated corn cobs and grape seeds, instead of the untreated ones.

In brief, the previous treatment of the support did not improve the ethanol production neither favoured the cells adhesion, being thus proved to be an unnecessary step for the ethanol production process. The no need of the support material treatment previous its use for the cells immobilization is an important advantage considering the simplicity of preparation, and mainly the economy of the process, since eliminates one stage from the global process, reducing the energy and chemicals consumption and also avoiding the mass losses that the treatment causes on the material structure.

3.3.3 Evaluation of the nutritional effect of the support materials on fermentation performance

A curious fact observed during the fermentation runs was the elevated formation of free biomass in the media containing the support materials (treated or not) when compared to the medium containing only free cells (Figure 3.2 E and F). Based on these results, experimental assays were performed aiming to evaluate if the support materials have contributed with

nutritional sources to the medium, which would have favoured the microorganism growth (Figure 3.3). In these assays, the cultivation medium was or not put in contact with the support material during 30 h previous the use in the fermentation runs, which were only performed with free cells in suspension.

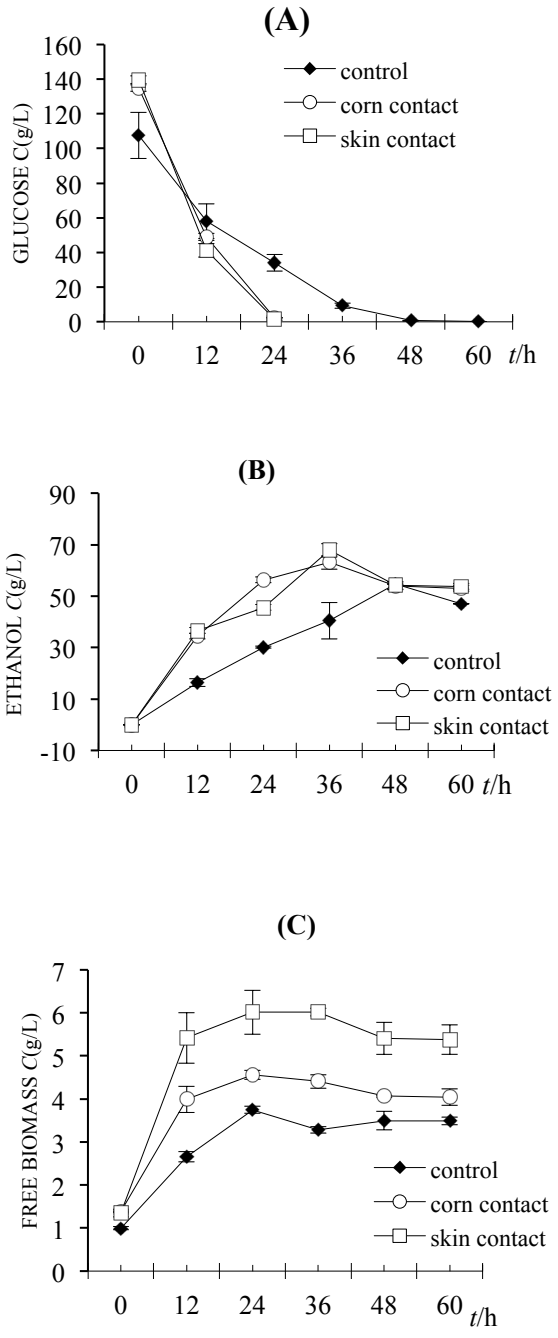

Figure 3.3. Time course of glucose consumption (A), ethanol production (B), and free biomass formation (C) during the fermentation runs using the medium with or without (control) previous contact with the support materials. Fermentation assays only with free cells in suspension.

Figure 3.3 clearly shows that the previous contact between the support materials and the fermentation medium favoured the glucose consumption and ethanol production by the yeast, as well as the biomass formation. Probably, some mineral or protein present in the materials' composition were solubilized to the fermentation medium and contributed for a better performance of the microorganism.

It is worth mentioning that, the ethanol production was faster in the samples with previous contact with the support (Figure 3.3 B), but it was slower than in the fermentations with immobilized cells (Figure 3.2 C and D). Similarly, glucose was consumed faster by immobilized cells (Figure 3.2 A and B) than by the free cells cultivated in the medium previously maintained in contact with the support materials (Figure 3.3 A). Such results allow concluding that the use of the natural materials, mainly grape skins and corn cobs, for the cells immobilization during the fermentation process is advantageous for two main reasons: 1) the materials allow the immobilization of high cell loads, which could be reused in other fermentation systems, and 2) the materials also provide nutrients to the medium, improving the yeast bioconversion performance.

3.3.4 Use of immobilized cell system under agitated conditions

In a last stage, the influence of the agitation on cells immobilization and ethanol production was evaluated (Figure 3.4).

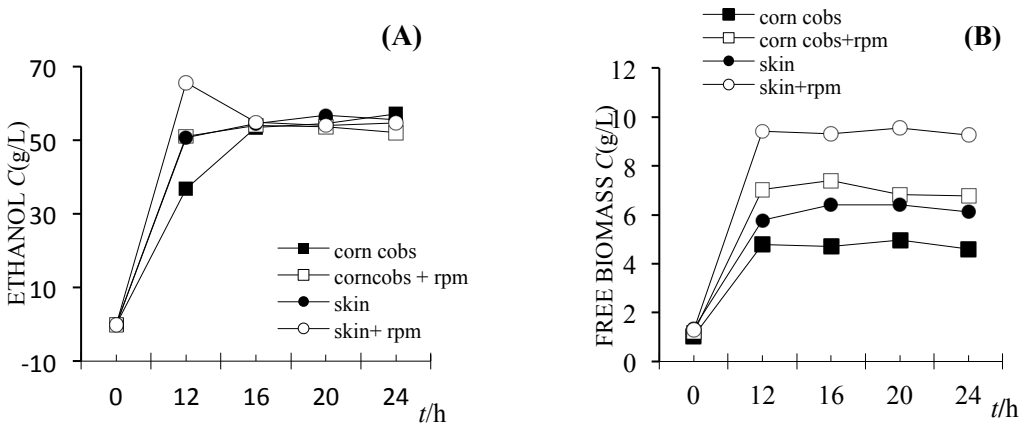

Figure 3.4. Time course of ethanol production (A), and free biomass formation (B) during the fermentation runs carried out with agitation (+rpm) and without agitation.

Use of agitated systems did not affect the ethanol production, which was similar to that obtained for the static systems. However, a strong negative influence of the agitation was observed on the cells adhesion to the supports. Under agitation, untreated corn cobs and grape skins were able to immobilize only 13.9 mg/g, and 10.3 mg/g, respectively (mass of cells per mass of support); values that correspond to approximately half of those obtained under static conditions. As a consequence of this lower immobilization results and the higher aeration of the

medium, the free cells concentration in these media was about two times higher than those observed under static conditions (Figure 3.4 B). Therefore, the ethanol production was not affected, but if it is desired the cells reuse in other fermentation operations, the use of immobilized cell systems under static conditions would be a better alternative.

3.4 Conclusions

Based on all the findings of this study it can be concluded that static fermentations using cells immobilized on untreated grape skins or corn cobs appear to be an interesting alternative to obtain an efficient ethanol production and high immobilized cells concentration. Such systems have potential to be successfully used in winemaking, since the support materials are cheap, available in large amounts and have food-grade purity. The use of grape skins, particularly, which is a by-product of the wine elaboration, could be of larger interest to obtain an integrated wine production process with by-product reuse. The performance of the fermentation for winemaking using cells immobilized in these two selected support materials, as well as the sensorial analysis of the obtained products will be the focus of our future studies.

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4. Operational stability of immobilized yeast cell system

White wine was produced with *S. cerevisiae* cells immobilized on grape skins, as well as with free cells. Seven repeated batch fermentations were carried out (batch 1 to batch 7). Then the immobilized support was stored for 30 d at 4 ^{o}C. After storage, three more fermentations were carried out (batch 8 to batch 10) with different concentrations of free SO_2 in the medium. The produced wines were subjected to chemical analysis by HPLC (ethanol, glycerol, sugars and organic acids) and by gas chromatography (major and minor volatile compounds); additionally colour (CIELab) and sensory analysis were performed. The fermentations with immobilized cells were much faster, *i.e.* advantageous over free cell fermentation. In terms of aromatic compounds, free cell fermentations were always equal to some of the batch immobilized cells fermentations. Support can be stored for at least one month without losing biological activity. The produced wines after storage were not found to be different from the wines before storage. The high doses of SO_2 had no influence on the overall aroma and quality of the produced wines.

The information presented in this chapter was submitted for publication.

Genisheva, Z., Mussatto, S., Vilanova, M., Teixeira, J.A. Oliveira, J.M. Consecutive batch fermentations of grape must with immobilized yeasts and assessment of biocatalyst storage and SO_2 concentration on wine quality.

4.1 Introduction

Cell immobilization systems used for alcoholic fermentations have various technological and economic advantages when compared with free cell systems, including the improvement of the productivity, the greater tolerance to inhibitory substances and the possibility of operating the processes in a continuous mode (Diviès and Cachon, 2005; Genisheva *et al*., 2011; Kourkoutas *et al*., 2004). It is well known that immobilized cells, compared to free cells, are more resistant against ethanol toxicity, acidity, extreme temperatures and some inhibitors like heavy metals, phenols and sulfur dioxide (Diviès and Cachon, 2005; Yajima and Yokotsuka, 2001).

The immobilization techniques can be divided into four categories: attachment to a support, entrapment in a porous matrix, cell aggregation and containment behind a barrier (Kourkoutas *et al*., 2004). The supports to be used can be organic or inorganic; however, it is considered that organic supports from natural origin, such as fruit pieces, can be easily accepted by the consumer (Genisheva *et al*., 2012; Kourkoutas *et al.*, 2004). Apple (Kourkoutas *et al*., 2002a), quince (Kourkoutas *et al*., 2002b), pear (Mallios *et al*., 2004), grape skins (Mallouchos *et al*., 2002) and dried raisin berries (Tsakiris *et al*., 2004a) have already been studied and presented advantages for application on an industrial scale, as they are of food grade purity and could reduce the cost of the process. Nevertheless, deeper studies on the immobilization practice must be done in order to ease the handling of the process and the use of this tool at the cellar (Kourkoutas *et al*., 2004; Vila-Crespo *et al*., 2010).

In fermentation processes with immobilized cells, the possibility of storage of the immobilized microorganisms for further use is an important aspect that must be taken into account (Diviès and Cachon, 2005; Genisheva *et al*., 2013; Kandylis *et al.*, 2010). Additionally, to be used in a winemaking process, the support must satisfy other prerequisites, such as: be abundant and cheap (Bakoyianis *et al*., 1992). In this context grape skins are hygienic, abundant and of a low cost, as it is a byproduct extensively generated in the wine industry. It is also a natural product from the vine, which supposedly will not interfere negatively on the final quality of the wine.

The aim of the present study was to evaluate the possibility to carry out consecutive alcoholic fermentations using *Saccharomyces cerevisiae* yeasts immobilized on grape skins to produce a white wine. Additionally, the stability of the immobilized biocatalyst after a storage period and the possible inhibitory effect of SO_2 were also studied. To assess the quality of the final products, physicochemical characteristics, colour, volatile compounds and sensory properties were evaluated.

4.2 Materials and Methods

4.2.1 Inoculum preparation

A commercial *Saccharomyces cerevisiae* strain (Lalvin QA23, Proenol) was used in the experiments. The inoculum was prepared by hydrating 300 mg/L of yeast in sterilized warm water (30 ^{o}C) for 30 min, according to manufacturer's instructions.

4.2.2 Support materials for cell immobilization

Grape skins were used as support material for cell immobilization. This support was supplied by a local winemaking company, being washed with distilled water and dried at 60 ºC until constant weight before use. Then, the support was sterilized for 20 min at 121 ^{o}C.

4.2.3 Fermentation assays

Seven alcoholic fermentations were carried out in consecutive batches (from batch 1 to batch 7). For the first batch, 50 g of dry grape skin were placed in 1 L of grape must and 300 mg of rehydrated yeast cells were added. The must/wine density was monitored daily and the fermentation was stopped when it was below 0.997 g/mL. After that, the support was recovered and washed with 500 mL of sterilized water and reused in the next batch. Free cell fermentations, with the same cellular concentration, were performed as controls. All experiments were performed at room temperature (≈ 20 °C), after adjusting SO_2 concentration to 30 mg/L, without agitation and in triplicate.

After batch 7, the supports with immobilized cells were washed with sterilized water (500 mL) and stored at 4 ºC for 30 d. Then, after storage of the immobilized biocatalysts, three more successive fermentation batches were performed. Batch 8 was carried out in the same conditions of batch 7. Batch 9 and batch 10 were conducted with increased concentration of free SO_2, respectively 60 mg/L and 90 mg/L.

Before bottling, all the produced wines were clarified by centrifugation (10 min, *RCF*=6000), and sulfur dioxide was adjusted to 30 mg/L. Wines were stored at 4 °C before analysis.

The following nomenclature was adopted: FC for fermentations with free cells and B1, B2, B3, B4, B5, B6, B7, B8, B9 and B10 for fermentations using immobilized cells.

4.2.4 Immobilized cells determination

Immobilized cells concentration was determined at the fermentations' end by dry weight, after washing the support with biocatalysts with 30 g/L NaOH solution, for 24 h, at 30 ºC and agitation rate of 120 min^{-1}, according to Genisheva *et al.* (2011). Free cells concentration in the fermentation medium was estimated by measuring the absorbance at 600 nm, which was

correlated to a calibration curve (dry weight × absorbance). Immobilized death/live yeasts were determined after detachment of the cells by vigorous agitation of 0.5 g of support with 30 g/L solution of NaCl, for 30 min. Then the liberated cells were further stained with methylene blue and the dead/live cells were counted on a Neubauer chamber.

4.2.5 General physicochemical analysis

Free SO_2 concentration and total acidy were measured by titration according to the methods OIV-MA-AS323-04A and OIV-MA-AS313-01, respectively (OIV, 2012a).

4.2.6 HPLC analysis

Glucose, fructose, ethanol, glycerol and organic acids (citric, tartaric, malic, succinic lactic and acetic) concentrations were determined according to Genisheva *et al*. (2013), by high performance liquid chromatography (HPLC) in a Jasco chromatograph equipped with a refractive index detector (Jasco 830-RI), an ultraviolet detector and a Varian Metacarb 67H column (300 mm × 6.5 mm) operated at 80 ºC. A 5 mmol/L H_2SO_4 aqueous solution was used as eluent at a constant flow rate of 0.3 mL/min.

4.2.7 Gas-Chromatographic analysis

Major volatile compounds were directly analysed after adding 410 μg of 4-nonanol (internal standard – IS) to 5 mL of wine. A Chrompack CP-9000 gas chromatograph equipped with a split/splitless injector, a flame ionization detector (FID) and a capillary column, coated with CP-Wax 57CB (50 m × 0.25 mm; 0.2 μm film thickness, Chrompack), was used. The temperatures of the injector and the detector were both set to 250 °C. The oven temperature was initially held at 60 °C, for 5 min, then programmed to rise from 60 °C to 220 °C, at 3 °C/min, and finally maintained at 220 °C for 10 min. The carrier gas was helium 4×(Praxair) at an initial flow rate of 1 mL/min (125 kPa at the head of the column). The analyses were performed by injecting 1 μL of sample in the split mode (15 mL/min).The quantification of major volatile compounds, after the determination of the detector response factor for each analyte, was performed with the software Star-Chromatography Workstation version 6.41 (Varian) by comparing retention times with those of pure standard compounds.

Minor volatile compounds were analysed by GC-MS after extraction of 8 mL of wine with 400 μL of dichloromethane, spiked with 3.28 μg of 4-nonanol (IS), according to the methodology proposed by Oliveira *et al.* (2006). A gas chromatograph Varian 3800 with a 1079 injector and an ion-trap mass spectrometer Varian Saturn 2000 was used. A 1 μL injection was made in splitless mode (30 s) in a Varian Factor Four VF-Wax ms column (30 m × 0.15 mm; 0.15 μm film thickness). The carrier gas was helium 4× (Praxair) at a constant flow rate of

1.3 mL/min. The detector was set to electronic impact mode with an ionization energy of 70 eV, a mass acquisition range from 35 *m*/*z* to 260 *m*/*z* and an acquisition interval of 610 ms. The oven temperature was initially set to 60 ^{o}C for 2 min and then raised from 60 ^{o}C to 234 ^{o}C at a rate of 3 ^{o}C/min, raised from 234 ^{o}C to 250 ^{o}C at 10 ^{o}C/min and finally maintained at 250 ^{o}C for 10 min. The temperature of the injector was maintained at 250 ^{o}C during the analysis time and the split flow was maintained at 30 mL/min. The identification of compounds was performed using the software MS Workstation version 6.9 (Varian) by comparing their mass spectra and retention indices with those of pure standard compounds. The minor compounds were quantified in terms of 4-nonanol equivalents only.

4.2.8 Colour analysis

The colour of the wines was assayed by the CIELab method, according to Genisheva *et al*. (2012), by measuring the absorbance between 380 nm and 770 nm (data pitch = 2 nm), using a Jasco UV/Vis V-560 spectrophotometer. The recorded data were processed by an algorithm using the program Matlab version r2010a, developed by the Science of Vision and Colour Laboratory, Department of Physics, University of Minho, to obtain the CIELab coordinates, L^*, $a^*$and b^*. These coordinates allowed the determination of other three parameters in the produced wines: saturation (C^*), variation in saturation (ΔC^*) and variation in lightness (ΔL^*), according to Almela *et al*. (1995). The following equations were used:

$$C^* = \sqrt{a^{*2} + b^{*2}} \qquad \text{(Equation 1)}$$

$$\Delta C^* = C^*_{\mathrm{x}} - \bar{C}^* \qquad \text{(Equation 2)}$$

$$\Delta L^* = L^*_{\mathrm{x}} - \bar{L}^* \qquad \text{(Equation 3)}$$

C_{x}^* and $L_{\mathrm{x}}^*$are the saturation and lightness of the wines produced by immobilized cells, and $\bar{C}^*$ and $\bar{L}^*$are the saturation and lightness, respectively of the reference wines, *i.e*. wines produced with free cells.

4.2.9 Sensory analysis

Ten tasting panellists (four male and six female), with ages between 40 and 50 years old and all of them having a long experience in sensory analysis, carried out the descriptive sensory analysis of wines, in two distinct sessions. In the first session, and to establish the descriptors of wines, the evaluation was performed using QDA methodology (Lawless and Heymann, 1998). Two training periods of 1 h were carried out, where judges generated descriptive terms to define the wines. In the second session, a constant volume of 30 mL of each wine was evaluated in wine-taster glasses at 12 ºC as described by the Norm ISO 3591 (1997). During the analysis, the

wine tasters scored the intensity of each attribute using a ten-point scale. Relative frequency (*F*), relative intensity (*I*) and geometric mean (*GM*) of the different descriptors were calculated for each wine. *GM* was calculated as the square root of the product between relative intensity and relative frequency, *i.e.* $GM/\% = \sqrt{I \times F} \times 100$. The descriptors were classified for each wine by using the *GM*, according to the Norm ISO 11035 (1994) which make possible the elimination of relatively low values. Consequently, only descriptors presenting *GM*> 15 % for at least one wine, were considered.

As complementary study, a triangle test was applied for determining whether a perceptible sensory difference exists between samples FC and B1 and between FC and B7 (Norm ISO 4120, 2004). For each analysis, two sets of samples were used: FC-B1-B1 and B1-FC-FC; FC-B7-B7 and B7-FC-FC.

4.2.10 Statistical analysis

The data were analysed using XLstat-Pro (Addinsoft, Paris 2009). To test significant differences among wines intensity analysis of variance (ANOVA) was applied.

As referred previously, three replicate assays were done for the batch fermentations. Accordingly, HPLC, GC, colour and general physicochemical analyses were done in triplicate, *i.e.* one per replicate. For sensory analysis, the three replicates were mixed before testing.

4.3 Results and Discussion

4.3.1 General characterization of fermentation assays

At the end of the fermentation assays the following measurements were carried out: pH, total acidity, free and immobilized cells concentrations, and percentage of immobilized death cells (Table 4.1). Multiple comparison analysis by Tukey´s test (p<0.05) was performed.

In general, the fermentation time diminished with the number of repeated batch fermentations, being initially 7 d, for FC and B1 assays, and tends to stabilize in 4 d, as reported for the later fermentations, B6 and B7. After storage (batch 8) the fermentation time increased to 5 d, but in batch 9 and batch 10 it diminished again to 4 d. A continuous decrease in the fermentation time (about one half after the 4th assay) for successive batch fermentations of glucose with *S. cerevisiae* immobilized in gluten pellets, have been already referred by Bekatorou *et al.* (2001); likewise, an acceleration of the alcoholic fermentation by yeasts immobilized in alginate gel beads has been reported (Diviès and Cachon, 2005). On the other hand, the unusual higher concentrations of SO_2 in the fermenting must seem not to affect the yeasts activity. Similar results were stated by Yajima and Yokotsuka (2001) when immobilized yeasts in double-layer gel beads of Ca-alginate are used. These authors showed an indubitable reduction of the time

needed to complete the alcoholic fermentation, after a previous adaptation of the immobilized yeast cells Additionally, as reported in other studies (Tsakiris *et al.*, 2004b; Kandylis *et al.*, 2010) the immobilized biocatalysts don´t show any loss of operational stability after the 10 batch fermentations. These features, associated to global end product quality, should be very important when an industrial process is planned.

Table 4.1. General characterization of fermentation assays: fermentation length time (*t*) and multiple comparison analysis (Tukey's test; $p<0.05$), including standard deviation (*sd*), for total acidity as tartaric acid (*TA*), pH, concentration of immobilized cells (X_{im}), free cells ($X_{f.cel}$), immobilized death cells (D_{im}) and total produced cells(X_t)

	t			*TA*		$X_{f.cel}$		X_{im}		D_{im}		X_t	
	d	pH	*sd*	g/L	*sd*	g/L	*sd*	mg/g	*sd*	%	*sd*	g/L	*sd*
FC	7	2.88^{c}	0.01	6.28^{c}	0.04	4.90^{a}	0.11	0.00	0.00	0.00	0.0	4.90^{c}	0.11
B1	7	3.07^{a}	0.01	6.58^{abc}	0.04	3.22^{bc}	0.06	24.20^{d}	6.35	9.3^{ab}	4.5	4.43^{c}	0.32
B2	5	2.83^{de}	0.02	5.50^{d}	0.04	3.78^{ab}	1.53	32.70^{d}	4.16	6.2^{ab}	2.0	5.41^{c}	1.34
B3	6	2.80^{e}	0.01	6.28^{c}	0.04	3.02^{bc}	0.13	43.33^{d}	2.24	5.6^{b}	0.9	5.18^{c}	0.11
B4	4	2.92^{b}	0.01	6.35^{bc}	0.04	3.20^{bc}	0.42	55.37^{d}	5.83	4.5^{b}	1.5	5.97^{bc}	0.56
B5	5	2.95^{b}	0.01	6.28^{c}	0.23	2.72^{bcd}	0.17	50.03^{d}	19.38	5.5^{b}	1.1	5.23^{c}	0.81
B6	4	2.86^{cd}	0.01	6.28^{c}	0.09	2.74^{bcd}	0.22	72.23^{cd}	9.76	8.5^{ab}	1.1	6.35^{ab}	0.68
B7	4	2.87^{c}	0.01	6.35^{bc}	0.24	2.47^{bcde}	0.17	87.47^{bcd}	5.13	4.5^{b}	1.5	6.83^{ab}	0.42
B8	5	2.85^{cd}	0.02	6.65^{ab}	0.04	0.95^{cde}	0.11	160.67^{ab}	8.14	8.5^{ab}	1.4	8.99^{ab}	0.52
B9	4	2.86^{cd}	0.00	6.65^{ab}	0.04	1.52^{de}	0.13	183.60^{a}	41.77	11.8^{a}	1.1	10.70^{a}	2.04
B10	4	2.93^{b}	0.03	6.85^{a}	0.09	1.10^{e}	0.16	178.43^{ab}	87.13	11.5^{a}	1.6	10.02^{ab}	4.38

a, b, c, d – values with the same letters mean no significant difference at 95 % confidence level.

Total acidity, determined as tartaric acid, varies between 6.28 g/L and 6.85 g/L, being the highest value found for wine produced in batch 10 (6.85 g/L). The pH values of the produced wines were found to be between 2.80 and 3.07.These results are in the normal range for white wines (Ribéreau-Gayon *et al*., 2006).

Concentrations of free cells have a tendency to diminish, from batch 1 to batch 7, reaching a value around two times less compared with the free cell assays. In contrast, the concentration of immobilized cells increased from batch 1 to batch 7, demonstrating stronger cell-cell or cell-support interactions. The total cell concentration also increased with the repeated batch fermentations (B1 to B7), being FC assays those with the lowest values. After the storage (4 ^{o}C, 30 d) of the support with immobilized cells (B8, B9 and B10 assays) free cells concentrations were much lower compared to batch 7 (Table 4.1). Additionally, the concentration of the immobilized cells increased two times, being the highest amount recorded for batch 9 (183.60 mg/g). The highest total concentrations of cells was also recorded for B9 assay (10.70 g/L), followed by B10 assay (10.02 g/L). Therefore, the previous storage of the

immobilized yeasts and the increased amount of sulfites in the assays B8 to B10 seem to promote the immobilization of free cells on the support. Batch 9 and batch 10 had the highest concentrations of immobilized cells but also had the highest percentage of death immobilized cells (11.8 % and 11.5 %, respectively), probably due to the high concentration of SO_2.

4.3.2 Ethanol, glycerol, sugars and organic acids

The concentrations of glucose, fructose, glycerol, ethanol and organic acids (citric, tartaric, malic, succinic, lactic and acetic), are shown in Table 4.2.

Glucose and fructose were present in low concentrations for all produced wines, thus confirming the completion of alcoholic fermentation; furthermore wines did not show significant differences ($p<0.05$). Glycerol is the most important by-product of the alcoholic fermentation. Normally, in wines, glycerol can be found in concentrations from 5 g/L to 15 g/L (Ribéreau-Gayon *et al*., 2006). In our study, the highest content of glycerol was recorded for wine from batch 1, which was found to be different ($p<0.05$) from all the other wines. Ethanol content is one of the main characteristics of the wine and is a key factor for its quality, giving body and viscosity (Ribéreau-Gayon *et al*., 2006). Ethanol may vary from 8 % vol. to 16 % vol., depending on the style of the wine and on the grape variety from witch is made (Ugliano and Henschke, 2009). In this study, ethanol concentrations vary from 11.2 % vol. (B8) to 12.1 % (B3), indicating that wines have a good strength. In regard to citric acid concentration, wine produced with free cells is significantly different ($p<0.05$) from the wines produced in the batch series using immobilized cells, showing the lowest value. Low concentrations of citric acid between 200 mg/L and 300 mg/L, are usual in wines (Costantini *et al*., 2009). Tartaric acid was the acid with the highest concentration in all the produced wines, which could be explained by the fact that this acid is usually found in high concentrations in grapes and do not undergo large changes during fermentation. Succinic and lactic acids (organic acids produced during the alcoholic fermentation) were present in wine B1 at concentrations significantly different with respect to the other wines. The concentration of acetic acid in the wines was lower than 1 g/L, showing that no bacterial contamination occurred during the grape must fermentation. Moreover, all the recorded values were always below the acceptable limit for white wines of 1.2 g/L (OIV, 2012b).

Even though statistical differences were found for the composition of the produced wines, those produced with free cells usually presented similarity with some of the wines produced with immobilized cells in successive batches; the highest differences were found between wines produced from free cells (FC) and the first batch fermentation (B1). This fact demonstrates that the main characteristics of wines produced with free cells and with immobilized cells are not so different. The fermentation time was the main parameter differentiating them (Table 4.1)

Table 4.2. Mean concentrations (*C*) and confidence limits (p = 0.05) for sugars, organic acids, ethanol and glycerol analysed by HPLC at the end of the alcoholic fermentation

compound	FC		B1		B2		B3		B4		B5		B6		B7		B8		B9		B10	
	C/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±	*C*/(g/L)	±
glucose	2.6^{a}	0.1	3.4^{a}	0.2	2.5^{a}	0.7	2.8^{a}	0.3	3.7^{a}	1.22	2.3^{a}	0.4	3.0^{a}	0.3	$3.1^{a}{}_{a}$	1.0	2.2_{b}	0.2	2.3_{b}	0.2	2.4_{b}	0.2
fructose	7.6^{ab}	1.1	3.9^{c}	0.2	8.2^{ab}	3.5	5.3^{bc}	2.1	10.0^{a}	3.94	3.2^{c}	1.1	7.9^{ab}	2.2	$7.7^{ab}{}_{a}$	5.6	1.4_{b}	0.2	1.6_{b}	0.2	3.3_{b}	0.3
glycerol	7.1^{b}	0.2	9.6^{a}	0.6	7.1^{b}	0.5	6.8^{b}	0.4	6.6^{b}	1.47	7.0^{b}	0.3	7.0^{b}	0.2	$6.9^{b}{}_{b}$	0.6	7.4_{a}	0.3	7.2_{ab}	0.5	7.4_{a}	0.2
ethanol	90.2^{ab}	2.9	94.6^{ab}	3.9	89.1^{b}	5.9	95.2^{a}	5.8	90.6^{ab}	8.61	92.4^{ab}	1.6	93.9^{ab}	4.7	$92.4^{ab}{}_{b}$	1.8	88.7_{c}	2.5	94.2_{a}	0.7	91.6_{b}	1.6
citric acid	0.3^{b}	0.0	0.6^{a}	0.1	0.6^{a}	0.0	0.6^{a}	0.0	0.6^{a}	0.10	0.6^{a}	0.0	0.6^{a}	0.0	$0.6^{a}{}_{b}$	0.0	0.7_{a}	0.1	0.5_{c}	0.0	0.6_{b}	0.0
tartaric acid	4.4^{bc}	0.1	5.4^{a}	0.2	4.2^{bc}	0.7	3.8^{cd}	0.2	4.0^{bcd}	0.30	4.6^{b}	0.4	4.5^{b}	1.1	$3.6^{d}{}_{c}$	0.7	5.3_{a}	0.5	4.4_{b}	0.9	5.1_{ab}	0.9
malic acid	2.0^{d}	0.1	3.2^{a}	0.1	2.3^{bc}	0.1	2.3^{bc}	0.2	2.1^{cd}	0.19	2.3^{b}	0.1	2.3^{bc}	0.2	$2.3^{bc}{}_{a}$	0.0	2.4_{a}	0.6	2.0_{b}	0.1	2.3_{a}	0.1
succinic acid	1.4^{bc}	0.1	2.5^{a}	0.3	1.39^{bc}	0.1	2.3^{ab}	1.1	2.3^{abc}	2.16	1.3^{c}	0.0	1.3^{c}	0.1	$1.3^{c}{}_{b}$	0.0	1.7_{a}	0.2	1.7_{a}	0.6	1.4_{ab}	0.1
lactic acid	1.5^{cd}	0.1	5.1^{a}	0.2	1.5^{d}	0.2	2.7^{b}	1.2	2.2^{bcd}	1.57	2.4^{bc}	0.8	2.6^{b}	1.0	$2.3^{bcd}{}_{b}$	0.1	5.4_{a}	3.7	4.0_{ab}	3.2	1.6_{b}	0.2
acetic acid	0.5^{c}	0.0	0.3^{d}	0.0	0.2^{e}	0.0	0.2^{e}	0.0	0.2^{e}	0.09	0.5^{bc}	0.1	0.5^{b}	0.1	$0.7^{a}{}_{b}$	0.1	0.2_{c}	0.1	0.2_{c}	0.0	1.0_{a}	0.2

a, b, c, d – values with the same letters mean no significant difference at 95 % confidence level; Superscript letters compare assays FC to B7; Subscript letters compare assays B7 to B10

The previous storage of the support with immobilized cells (wine B8) and the higher concentrations of SO_2 in the medium (wines B9 and B10) yielded higher concentrations of glycerol in the wine when compared to the wine B7. This is in accordance with Ribéreau-Gayon *et al*. (2006) who observed an increase of glycerol concentration in wines, as high as 20 g/L, when high concentrations of SO_2 were applied. The storage of the immobilized support may negatively influence the ethanol production, while the use of high SO_2 concentrations seems to have no influence over its production. Wines B8 had higher amounts of citric, tartaric, succinic and lactic acids than B7 wines. However, the concentrations of these organic acids diminished in the subsequent fermentations (batches 9 and 10). The concentration of acetic acid was lower in wines produced in batch 8; in contrast, the highest concentration of free SO_2 in the must of batch 10 (90 mg/L) proportioned wines with the highest concentrations of acetic acid. For wines B10, acetic acid (1.03 g/L) almost reached the acceptable limit for white wines of 1.2 g/L (OIV, 2012b).

4.3.3 Major volatile compounds

Table 4.3 shows the 8 major volatile compounds identified in the produced wines. As a whole, statistical significant differences were found between wines, except for 1-propanol.

Acetaldehyde was found in all the samples in concentrations higher than its orthonasal perception threshold of 10 mg/L, and might give "overripe apple" notes to wines (Chaves *et al*., 2007; Moreno *et al*., 2005). The highest concentration of this aldehyde was observed in wines produced with free cells (26.5 mg/L). This fact is in agreement with the results published by Tsakiris *et al*. (2004b) who also observed higher amounts of acetaldehyde in wines produced with free cells; nevertheless, the obtained values were lower than those detected by Kourkoutas *et al.* (2002a) in wines produced with cells immobilized on quince (106 mg/L).

Ethyl acetate was found in all produced wines in concentrations above its perception threshold of 12.3 mg/L (Escudero *et al*., 2004), contributing to the "pineapple" and "nail polish" character of wines (Chaves *et al*., 2007). The highest concentrations were recorded for B7 wine (45.6 mg/L), which was significantly different from the others.

Immobilized cell fermentations from batch 1 to batch 7 presented slightly higher levels of methanol (57.7 mg/L to 189.0 mg/L) than those observed for white wines produced with cells immobilized on grape pomace (Genisheva *et al*., 2012) and on quince (Kourkoutas *et al*., 2002a). Nevertheless, all the assays contained methanol in concentrations below its limit permitted for consumption of 250 mg/L (OIV, 2012b). Methanol results from the pectins of the skin of the grapes that undergoes an enzymatic conversion (Ribéreau-Gayon *et al*., 2006). Since the fermenting must was in contact with the grape skins for long and repeated time periods, high

amounts of methanol could be found in the product. However, no differences were found between the wines produced with immobilized cells and with free cells.

Table 4.3. Mean concentrations (*C*), confidence limits ($p = 0.05$) and aroma perception thresholds (*PT*) of the major volatile compounds at the end of alcoholic fermentation

	FC		**B1**		**B2**		**B3**		**B4**		**B5**		**B6**		**B7**		**B8**		**B9**		**B10**		
	C/(mg/L)	±	*C*/(mg/L)	±	*C*/(mg/L)	±	*C*/(mg/L)	±	*C*/(mg/L)	±	*C*/(mg/L)	±	*C*/(mg/L)	±	*C*/(mg/L)	±	*C*/(mg/L)	±	*C*/(mg/L)	±	*C*/(mg/L)	±	*PT*/(mg/L)
acetaldehyde	26.5^{a}	20.5	15.0^{bc}	8.2	23.7^{ab}	4.5	15.3^{bc}	9.8	12.5^{bc}	5.2	10.8^{c}	3.4	25.2^{ab}	3.4	$20.1^{abc}{}_{a}$	5.0	11.8_{b}	1.7	9.9_{b}	5.9	12.4_{b}	2.7	10^{A}
ethyl acetate	31.8^{bc}	13.2	35.6^{abc}	8.2	30.7^{c}	9.8	35.9^{abc}	6.2	36.1^{abc}	2.8	43.5^{ab}	18.8	41.1^{abc}	10.4	$45.6^{a}{}_{a}$	7.4	40.4_{a}	14.6	29.7_{a}	21.7	36.8_{a}	22.4	12.3^{B}
methanol	96.9^{ab}	55.2	131.4^{ab}	42.7	145.5^{ab}	111.2	137.6^{ab}	76.7	189.0^{a}	150.3	145.7^{ab}	137.8	159.6^{ab}	154.3	$57.7^{b}{}_{c}$	26.7	75.1_{b}	12.9	77.3_{b}	6.7	162.2_{a}	4.9	668^{A}
1-propanol	26.5^{a}	9.5	32.7^{a}	13.6	31.7^{a}	10.8	34.7^{a}	15.7	35.2^{a}	19.5	38.2^{a}	19.1	36.8^{a}	16.0	$35.8^{a}{}_{a}$	30.7	25.7_{a}	11.5	22.4_{a}	9.7	26.3_{a}	16.3	830^{A}
2-methyl-1-propanol	33.7^{c}	3.9	34.6^{c}	10.7	33.4^{c}	8.9	45.9^{bc}	11.8	53.1^{abc}	15.6	70.7^{a}	8.2	54.2^{abc}	15.2	$60.0^{ab}{}_{a}$	44.3	71.2_{a}	23.3	61.8_{a}	26.3	61.9_{a}	32.8	40^{A}
2-methyl-1-butanol	35.8^{ab}	1.5	45.3^{a}	9.2	37.0^{ab}	10.4	33.0^{ab}	4.3	29.5^{b}	10.3	31.3^{b}	17.3	26.6^{b}	9.2	$25.8^{b}{}_{a}$	21.6	39.5_{a}	15.0	29.9_{a}	14.9	26.2_{a}	12.8	
3-methyl-1-butanol	147.6^{a}	12.8	159.5^{a}	28.1	148.9^{a}	41.0	155.4^{a}	39.0	153.0^{a}	49.5	182.0^{a}	58.6	148.2^{a}	58.3	$149.2^{a}{}_{a}$	109.5	194.7_{a}	75.1	159.2_{a}	81.7	149.2_{a}	76.7	30^{A}
2-phenylethanol	38.6^{ab}	27.0	42.3^{ab}	36.7	52.9^{a}	12.4	31.0^{ab}	24.3	26.6^{b}	19.6	19.8^{b}	8.7	22.1^{b}	14.2	$30.0^{ab}{}_{a}$	16.8	38.6_{a}	27.3	27.0_{a}	16.3	23.5_{a}	7.1	14^{C}
total higher alcohols	**282.2**	31.6	**314.4**	50.2	**303.9**	46.2	**300.0**	50.2	**297.4**	59.7	**342.0**	65.1	**287.9**	64.6	**300.8**	125.1	**369.7**	85.4	**300.3**	89.2	**287.1**	86.2	

a, b, c, d – values with the same letters mean no significant difference at 95 % confidence level between fermentation essays; Superscript letters compare assays FC to B7; Subscript letters compare assays B7 to B10; **A-** Moreno *et al*., 2005; **B-** Escudero *et al*., 2004; **C-** Ferreira *et al*., 2000

Respecting to higher alcohols, all the produced wines present similar levels of these compounds, although FC and B10 assays seemed to have lower concentrations, around 300 mg/L. Although higher alcohols, individually, do not give pleasant notes to the wine (except 2-phenylethanol), together they can positively contribute to the overall aroma. Low concentrations of higher alcohols (below 300 mg/L to 400 mg/L) have been reported to positively contribute to the overall aroma of wines (Rapp and Versini, 1995). On the other hand, higher concentration of these compounds can bring "strong" and "pungent" notes (Nykänen, 1986), depending, however, on the global wine composition.

Individually, 2-methyl-1-propanol, 3-methyl-1-butanol and 2-phenyletanol were present in the wines concentrations above their perception thresholds of 40 mg/L, 30 mg/L and 14 mg/L, respectively (Escudero *et al*., 2004; Moreno *et al*., 2005); 2-methyl-1-propanol and 3-methyl-1-butanol may contribute to the "spirituous", "fusel" and "nail polish" odour notes of wines (Siebert *et al*., 2005), mainly for assays B3 to B7. Moreover, the presence of 2-phenylethanol in the samples (19.8 mg/L to 52.9 mg/L), above its perception threshold, may give "rose" and "sweetish" nuances to the wines (Siebert *et al*., 2005).

The storage of the support with immobilized cells and the higher doses of free SO_2 did not influence the production of major volatile compounds, with the exception of acetaldehyde and methanol. Acetaldehyde concentration diminished after the previous storage of the immobilized support, as well as with the higher concentrations of SO_2 added.

4.3.4 Minor volatile compounds

Table 4.4 shows a total of 24 minor volatile compounds that were identified and quantified by GC-MS which belongs to different chemical groups including ethyl esters, acetates, terpenols, C_{13}-norisoprenoids, volatile phenols and volatile fatty acids.

Ethyl butyrate, ethyl hexanoate and ethyl octanoate were found in concentrations markedly above their perception thresholds, respectively 20 μg/L, 14 μg/L and 5 μg/L, in all the produced wines. Similar fact occurred for ethyl decanoate, but only for some samples. Under these conditions, these four compounds may bring "fruity" (apple, papaya) and "sweetish" notes to the wines (Escudero *et al*., 2004; Meilgaard, 1975).

In all wines, isoamyl acetate was found in concentrations much higher than its odour perception threshold of 30 μg/L (Moreno *et al*., 2005), thus bringing "banana" notes to the overall aroma of the wines (Escudero *et al*., 2004; Genisheva *et al*., 2012). The 2-phenyl ethyl acetate was also found in all the wines in concentration levels above its perception threshold of 250 μg/L (Moreno *et al*., 2005) bringing "roses" and "flowery" notes to the wines (Escudero *et al*., 2004; Meilgaard, 1975). Wines produced with free cells had the lowest total concentration

of acetates when compared to wines produced with immobilized cells. Moreover, the total concentration of acetates seemed to increase from batch 1 to batch 6.

Table 4.4. Mean concentrations (*C*), confidence limits ($p = 0.05$) and aroma perception threshold (*PT*) of the minor volatile compounds at the end of alcoholic fermentation

	FC		B1		B2		B3		B4		B5		B6		B7		B8		B9		B10		*PT*/
	C/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	(μg/L)
Ethyl esters																							
ethyl butyrate	95.0^{ab}	16.7	89.6^{b}	17.5	139.8^{ab}	51.3	187.2^{a}	197.5	102.9^{ab}	26.8	100.5^{ab}	40.9	120.3^{ab}	7.9	120.2^{ab}_{a}	91.0	141.5_{a}	19.7	68.4_{b}	6.8	121.2_{a}	10.7	20^{A}
ethyl hexanoate	358.0^{a}	112.5	366.0^{a}	37.1	415.5^{a}	86.7	475.5^{a}	132.2	402.9^{a}	43.2	379.2^{a}	209.9	482.6^{a}	14.2	433.4^{a}_{ab}	191.7	344.6_{bc}	28.3	254.9_{c}	34.1	488.9_{a}	47.7	14^{B}
ethyl octanoate	350.4^{a}	39.8	311.4^{a}	28.2	365.5^{a}	120.8	449.0^{a}	98.3	426.3^{a}	56.2	380.3^{a}	182.0	431.3^{a}	132.0	357.1^{a}_{a}	205.8	252.6_{ab}	13.8	196.0_{b}	29.7	349.3_{a}	50.9	5^{B}
ethyl decanoate	270.7^{ab}	118.9	169.5^{b}	65.9	206.8^{ab}	117.8	244.4^{ab}	59.8	282.5^{a}	44.2	244.2^{ab}	89.2	251.1^{ab}	29.0	182.7^{ab}_{a}	135.2	122.0_{ab}	16.7	78.0_{b}	28.6	124.3_{ab}	42.9	200^{B}
total	**1074.1**	169.3	**936.5**	82.6	**1127.6**	196.5	**1356.1**	264.0	**1214.6**	87.7	**1104.2**	294.6	**1285.3**	136.1	**1093.4**	325.1	**860.7**	40.7	**597.3**	53.9	**1083.7**	82.6	
Acetates																							
Isoamyl acetate	517.9^{c}	174.9	698.8^{bc}	79.1	744.8^{bc}	113.5	889.6^{abc}	339.1	887.6^{abc}	181.9	972.4^{ab}	452.9	1209.5^{a}	40.2	1092.1^{a}_{ab}	586.0	871.2_{bc}	12.6	630.3_{c}	118.6	1398.7_{a}	337.0	30^{C}
hexyl acetate	27.2^{c}	8.6	28.6^{c}	8.5	45.3^{abc}	11.6	48.9^{ab}	11.1	61.6^{a}	27.2	48.2^{ab}	30.8	61.3^{a}	8.5	40.4^{bc}_{ab}	12.4	42.7_{a}	9.0	25.9_{b}	11.8	40.7_{ab}	16.5	1000^{D}
2-phenylethyl acetate	497.4^{a}	214.4	578.4^{a}	20.7	711.3^{a}	327.3	599.0^{a}	105.7	695.8^{a}	318.5	579.0^{a}	233.2	638.2^{a}	133.8	532.2^{a}_{ab}	271.9	508.8_{b}	103.0	479.1_{b}	72.6	708.0_{a}	154.7	250^{C}
total	**1042.5**	276.8	**1305.8**	82.2	**1501.4**	346.6	**1537.5**	355.4	**1645.0**	367.8	**1599.6**	510.3	**1909.0**	140.0	**1664.7**	646.1	**1422.7**	104.2	**1135.3**	139.6	**2147.4**	371.2	
Terpenols																							
linalool	104.6^{a}	13.3	74.4^{c}	11.0	91.1^{abc}	2.9	93.2^{abc}	3.2	96.5^{ab}	4.8	81.3^{bc}	36.3	90.3^{abc}	8.5	79.7^{bc}_{ab}	34.8	86.8_{ab}	2.9	69.1_{b}	5.1	94.1_{a}	5.1	25.2^{B}
HO-trienol	24.4^{a}	8.6	23.4^{a}	3.0	20.9^{a}	5.3	18.1^{a}	0.4	18.2^{a}	1.1	16.4^{a}	6.8	17.9^{a}	1.8	16.6^{a}_{ab}	8.6	15.1_{ab}	2.6	13.9_{b}	3.5	19.9_{a}	0.5	110^{E}
α-terpineol	40.0^{a}	12.7	28.0^{b}	3.7	39.2^{a}	5.6	38.4^{ab}	1.5	40.8^{a}	5.0	33.9^{ab}	14.2	36.9^{ab}	3.5	34.0^{ab}_{b}	14.8	38.5_{ab}	3.9	30.7_{b}	2.9	42.6_{a}	3.9	250^{B}
citronellol	12.6^{a}	6.2	6.9^{b}	0.5	8.5^{b}	0.8	7.1^{b}	1.8	8.2^{b}	0.7	6.7^{b}	1.5	5.9^{b}	1.6	5.7^{b}_{c}	2.8	16.1_{a}	1.9	11.3_{b}	0.3	11.6_{b}	1.4	100^{C}
nerol	4.4^{a}	2.1	3.9^{a}	0.8	4.3^{a}	2.1	3.7^{a}	0.7	4.0^{a}	0.4	3.2^{a}	1.1	3.4^{a}	0.3	3.1^{a}_{c}	1.2	5.1_{a}	0.9	3.1_{bc}	0.1	4.0_{b}	0.7	400^{F}

Table 4.4. Mean concentrations (C), confidence limits (p = 0.05) and aroma perception threshold (PT) of the minor volatile compounds at the end of alcoholic fermentation (cont.)

	FC		B1		B2		B3		B4		B5		B6		B7		B8		B9		B10		*PT*/
	C/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	(μg/L)
geraniol	37.1^{a}	11.1	27.3^{ab}	10.2	33.6^{a}	8.0	34.5^{a}	18.3	18.3^{b}	2.5	16.1^{b}	7.0	32.2^{a}	5.8	$30.3^{a}{}_{b}$	10.2	29.7_{b}	5.0	24.5_{b}	0.8	38.6_{a}	10.3	36^{G}
total	**223.1**	24.0	**163.9**	15.8	**197.6**	11.7	**195.0**	18.7	**186.0**	7.5	**157.6**	40.2	**186.6**	11.1	**169.4**	40.2	**191.3**	7.7	**152.6**	6.9	**210.8**	12.2	
C_{13}-norisoprenoids																							
β-damascenone	2.9^{a}	1.1	2.2^{a}	1.0	2.3^{a}	1.1	2.7^{a}	1.4	3.2^{a}	1.9	2.1^{a}	1.7	2.6^{a}	1.5	$1.9^{a}{}_{b}$	0.6	2.6_{a}	0.6	1.4_{b}	0.2	1.8_{b}	0.6	0.05^{A}
3-hydroxy-β-damascone	5.9^{ab}	0.9	7.1^{a}	2.6	7.7^{a}	2.6	6.0^{ab}	0.7	6.8^{a}	0.8	4.7^{bc}	0.9	4.9^{bc}	1.0	$3.5^{c}{}_{b}$	2.2	3.6_{b}	1.2	2.6_{b}	0.9	5.2_{a}	0.6	
3-oxo-α-ionol	5.9^{ab}	4.7	8.1^{a}	1.9	7.0^{ab}	1.1	4.7^{bc}	0.4	5.5^{abc}	2.3	3.1^{c}	2.4	6.2^{ab}	2.1	$4.8^{bc}{}_{b}$	1.8	5.2_{a}	1.1	1.4_{bc}	0.2	4.5_{b}	0.4	
total	**14.7**	4.9	**17.4**	3.4	**17.0**	3.0	**13.4**	1.6	**15.5**	3.1	**9.9**	3.1	**13.7**	2.8	**10.2**	6.4	**11.4**	1.7	**5.4**	0.9	**11.5**	0.9	
Volatile phenols																							
4-vinylguaiacol	404.7^{a}	163.6	72.2^{b}	5.8	128.0^{b}	12.2	133.6^{b}	11.3	142.2^{b}	17.7	131.2^{b}	42.9	141.1^{b}	26.1	$122.6^{b}{}_{a}$	49.4	75.5_{b}	8.1	69.0_{b}	11.3	102.6_{a}	4.7	130^{H}
4-vinylphenol	353.6^{a}	141.2	79.2^{c}	5.3	136.8^{bc}	21.0	133.7^{bc}	13.1	153.6^{b}	49.1	136.3^{bc}	51.2	146.7^{bc}	37.2	$130.5^{bc}{}_{a}$	57.7	75.4_{b}	9.4	72.0_{b}	14.3	109.4_{a}	6.4	180^{H}
total	**758.3**	216.1	**151.4**	7.9	**264.8**	24.3	**267.3**	17.3	**295.8**	52.2	**267.5**	66.8	**287.8**	45.4	**253.1**	76.0	**150.9**	12.4	**141.0**	18.2	**212.0**	7.9	
Volatile fatty acids																							
butanoic acid	92.2^{a}	38.2	14.7^{b}	10.7	9.2^{b}	0.6	8.1^{b}	4.0	9.7^{b}	3.2	8.1^{b}	1.9	10.4^{b}	5.2	$8.7^{b}{}_{b}$	4.6	21.8_{a}	2.1	9.6_{b}	1.9	8.9_{b}	0.2	173^{B}
hexanoic acid	308.5^{a}	111.8	348.0^{a}	69.7	398.1^{a}	74.5	353.3^{a}	65.3	412.4^{a}	130.3	316.6^{a}	118.9	394.2^{a}	106.4	$331.9^{a}{}_{a}$	159.8	266.1_{ab}	10.3	177.8_{b}	36.6	348.9_{a}	72.1	420^{B}
octanoic acid	1367.9^{b}	126.6	927.2^{c}	267.5	1519.1^{ab}	222.1	1521.6^{ab}	229.0	1785.4^{a}	81.2	1394.2^{ab}	512.0	1748.4^{ab}	353.2	$1508.8^{ab}{}_{a}$	624.7	751.8_{b}	143.1	623.3_{b}	88.1	1336.9_{a}	265.9	500^{B}
decanoic acid	1267.4^{a}	484.2	167.4^{c}	60.9	355.0^{bc}	153.4	474.5^{bc}	83.8	667.0^{b}	179.5	494.6^{bc}	164.7	682.7^{b}	115.6	$616.1^{b}{}_{a}$	262.3	92.4_{c}	25.7	115.3_{c}	17.5	282.8_{b}	120.4	1000^{B}
2+3-methyl butanoic acids	91.8^{b}	28.5	161.0^{a}	16.9	95.9^{b}	41.6	59.6^{cd}	10.1	70.9^{bc}	40.9	48.4^{cd}	14.8	47.8^{cd}	12.0	$40.3^{d}{}_{c}$	18.5	127.1_{a}	17.6	60.6_{b}	12.4	55.6_{bc}	11.5	33.4^{B}
total	**3127.8**	515.0	**1618.3**	283.8	**2377.3**	283.1	**2417.1**	252.7	**2945.4**	239.7	**2261.9**	551.0	**2883.5**	386.8	**2505.8**	696.4	**1259.2**	146.8	**986.6**	97.8	**2033.1**	300.9	

a, b, c, d – values with the same letters mean no significantdifference at 95 % confidence level between fermentation assays;nd-notdetected;Superscript letters compare assays FC to B7, Subscript letters compare assays B7 to B10; **;** **A**- Guth, 1997; **B**- Ferreira *et al*., 2000; **C**- Moreno *et al*., 2005; **D**-Chaves *et al*., 2007; **E**-Simpson, 1979; **F**-Ribéreau-Gayon *et al*., 2006; **G**- Escudero *et al*., 2004; **H**- Boidron *et al*., 1988

Terpenols were found in similar concentrations in all the wines. This could be explained by the fact that all assays were carried out with the same grape must, and terpenols are part of the varietal aroma of grapes (Genisheva and Oliveira, 2009; Oliveira *et al.*, 2008). In all produced wines, linalool was in concentrations above its perception threshold of 25 µg/L (Ferreira *et al*., 2000), thus bringing "flower" and "lavender" notes to the wines (Chaves *et al*., 2007). Geraniol, in free cell assays, was also found above its perception threshold of 36 µg/L (Escudero *et al*., 2004), bringing "flower" notes to the overall aroma of the wines (Ugliano and Moio, 2008).

The produced wines showed comparable levels of C_{13}-norisoprenoids, except for B9 assay. Similarly, β-damascenone did not show significant differences, but it was always above its perception threshold of 0.05 µg/L (Guth, 1997), thus bringing "sweet" and "apple" notes to the wines (Escudero *et al*., 2004).

Wines produced with immobilized cells (B1 to B7) did not show statistically significant differences ($p<0.05$) regarding the concentration of volatile phenols, particularly 4-vinylguaiacol. Nevertheless, the assay with free cells showed to be different, recording the highest concentration for this compound, 404.7 µg/L. Furthermore, this phenol was found in concentrations above its perception threshold of 130 µg/L (Boidron *et al.*, 1988) in several assays using immobilized cells, bringing "spice" and "wood" characteristic to the wines (Ugliano and Moio, 2008). Free cell fermentations also showed the highest value of 4-vinylphenol, and this compound was found in concentrations above its perception threshold of 180 µg/L (Boidron *et al*., 1988). Assays with free cells had two to three times higher total concentrations of volatile phenols than assays with immobilized cells. The volatile phenols 4-vinylguaiacol and 4-vinylphenol are produced during fermentation by the ability of *Saccharomyces cerevisiae* to decarboxylate hydroxycinnamic acids (Chatonnet *et al.*, 1993). The lower concentrations of volatile phenols in the assays with immobilized cells might be explained by the hypothetically modifications of the cell metabolism.

Octanoic acid and 2+3-methylbutanoic acid seem to have decisive influence on the aroma of all wines, since perception thresholds are, respectively, 500 µg/L and 33.4 µg/L (Ferreira *et al*., 2000). They may bring "cheese" and "rancid" notes to the overall aroma of the wines (Escudero *et al.*, 2004; Genovese *et al*., 2007). Moreover, hexanoic acid may influence the aroma of the produced wines since the determined concentrations are near the perception threshold. Additionally, the concentration of decanoic acid in the free cell assays (1267.4 µg/L) was above its perception threshold of 1000 µg/L. Wines produced from free cells recorded the highest total concentrations of volatile fatty acids.

The storage of support with the immobilized cells seems to influence negatively the production of ethyl esters, acetates, fatty acids and volatile phenols. In contrast, terpenic compounds and C_{13}-norisoprenoids had higher concentrations in wines produced with previously stored immobilized supports (B8). The higher concentration of SO_2 (60 mg/L or 90 mg/L) present in the grape must did

not have a strong influence over the minor volatile compounds, except for esters and acetates. For the majority of minor volatile compounds, the recorded concentrations for B9 wines were lower than those determined in B10 wines. This demonstrates the adaptation of immobilized cells to the higher concentration of SO_2 present in the grape must.

4.3.5 Colour analysis

Colour analysis of the wines (not performed for B8, B9 and B10 assays) was carried out using the CIELab method, with the determination of the coordinates L^*, a^* and b^*. Furthermore, in order to compare the wines, variation in lightness, ΔL^*, and variation in saturation, ΔC^*, were also determined (Table 4.5). The results obtained for the coordinates L^*, a^* and b^*, as well as for saturation C^*, showed significant differences between wines in terms of the colour parameters (Tuckey´s test). Wines produced with free cells had higher values for the brightness $L^*$and lower values for saturation C^*, revealing lower colour intensity. Moreover the parameter a^* had higher values, while parameter b^* had lower values, which indicate a yellowy-greenish colour. According to the colour parameters, wines produced in batches 6 and 7 were more similar to wines produced with free cells, than to the others. The wines from batch 1 had the highest colour intensity (lower values of L^*) as well as increased colour saturation (highest values of C^*), compared to the other produced wines.

Table 4.5.CIELab coordinates, including confidence limits (p = 0.05), and the calculated values for C^*, $\Delta L^*$and ΔC^*

	L^*	±	a^*	±	b^*	±	C^*	±	ΔC^*	ΔL^*
FC	94.0^{a}	0.4	-0.7^{a}	0.0	9.2^{d}	0.3	9.2^{d}	0.3	0.0	0.0
B1	88.0^{e}	1.5	-2.3^{d}	0.2	33.6^{a}	5.3	33.7^{a}	5.3	24.5	-6.0
B2	89.1^{e}	0.6	-1.0^{bc}	0.2	15.4^{b}	0.7	15.4^{b}	0.7	6.2	-4.9
B3	92.6^{bcd}	0.6	-0.9^{abc}	0.1	11.7^{c}	0.7	11.7^{c}	0.7	2.5	-1.4
B4	92.1^{cd}	1.6	-0.9^{abc}	0.2	11.8^{c}	0.5	11.9^{c}	0.5	2.7	-1.9
B5	91.6^{d}	1.7	-0.8^{ab}	0.3	11.5^{cd}	1.0	11.5^{cd}	1.0	2.3	-2.4
B6	93.5^{ab}	0.9	-1.0^{abc}	0.2	10.3^{cd}	1.5	10.4^{cd}	1.5	1.1	-0.5
B7	93.4^{abc}	1.1	-1.2^{c}	0.6	10.1^{cd}	1.1	10.1^{cd}	1.0	0.9	-0.56

a, b, c, d – values with the same letters mean no significant difference at 95 % confidence level; L^*, a^*, b^*– CIELab coordinates, C^* – saturation of colour, ΔC^*– variation of saturation, ΔL^*– variation of lightness.

Figure 4.1 shows the differences in colour of the produced wines, using a graphical representation of the variation in lightness, ΔL^*, as function of variation in saturation, ΔC^*, which reduces the CIELab coordinates into a two-dimensional colour space (Almela *et al*., 1995). Thus, the deviations in the colour of the wines produced by the immobilized cells, compared to those produced with free cells, could be observed. It was found that the wines produced in the first batch fermentation with immobilized cells (B1) had darker colour than those produced in the second fermentation (B2), due to the lower values of ΔL^*. In general the values of ΔL^*increase from batch 1 to batch 7, showing that

wines became brighter in that direction. As the number of successive batch fermentations increases the coloured compounds released from the grape skins diminished and the colour of the wines tended to stabilize becoming more similar to those produced with free cells. This fact was previously reported by Genisheva *et al*. (2012).

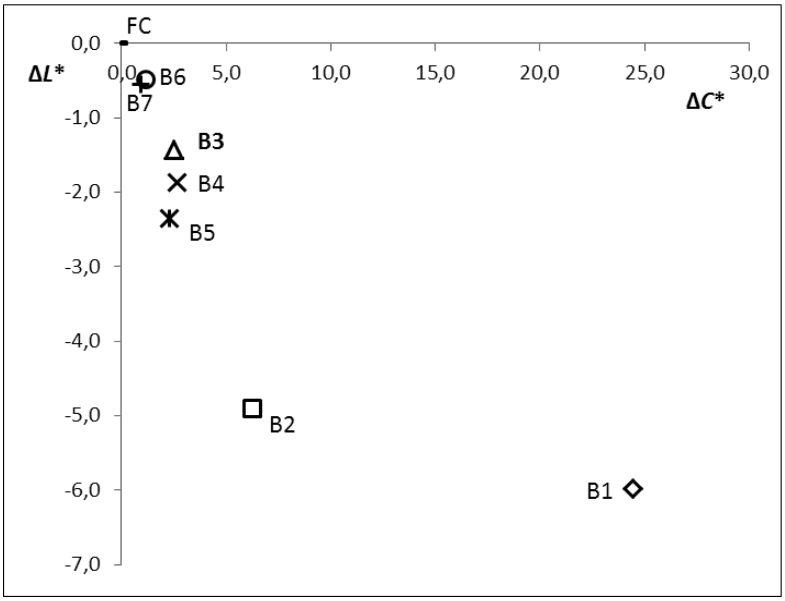

Figure 4.1. Variation of saturation, ΔC^*, and variation of lightness, ΔL^*, of wines produced using immobilized yeasts (batch series B1 to B7) and with free cells (FC).

4.3.6 Sensory analysis

An experienced panel performed the sensory characterization of the wines considered in this study (FC and B1 to B7). The panel generated a total of 29 descriptors from wines: 20 for aroma and 9 for taste; additionally, a global value was attributed (Table 4.6).Then, the geometric mean (*GM*) was determined in order to reduce the number of descriptors. Accordingly, Table 4.7 only shows the *GM* for the selected descriptors, *i.e.*10 for aroma and 8 for taste. The used QDA methodology permitted to take into account descriptors which were rarely mentioned but which are very important in terms of the perceived intensity, and descriptors with a low perceived intensity but which are mentioned often (Dravnieks and Bock, 1978).

Table 4.6. Descriptors of wines generated by the expert tasting panel

Descriptors	
Aroma	**Taste**
Intensity	Quality
Quality	Sweet
Dry fruit	Salty
Fruity	Acid
Vegetal	Bitter
Stone fruit	Body
Toast bread	Persistence

Table 4.6. Descriptors of wines generated by the expert tasting panel (cont.)

Descriptors	
Aroma	**Taste**
Walnut	Astringency
Apple	Spicy
Flower	
Honey	**Global value**
Pear	
Plum	
Liquorice	
Pepper	
Mineral	
Peach	
Citric	
Tropical	
Tobacco	

According to ANOVA (Table 4.7), performed on the individual intensity scores of the selected attributes, the effect of the wine assay was significant for the following aroma descriptors: intensity, toast bread, apple and honey. Taste descriptors and global value were not affected by the type of wine elaboration.

Table 4.7. ANOVA for the selected descriptors performed on wines produced with free (FC) and immobilized cells (B1 to B7)

Descriptors		FC	B1	B2	B3	B4	B5	B6	B7	Sig.
Aroma	Intensity	73.2^{b}	77.0b	60.9^{a}	70.7^{ab}	72.0^{ab}	74.5b	75.7^{b}	69.4^{ab}	*
	Quality	63.8	69.4	65.3	68.0	69.4	70.7	69.4	69.4	ns
	Dry fruit	22.2	0.0	24.9	0.0	0.0	9.6	12.4	19.3	ns
	Fruity	11.1	0.0	0.0	23.6	13.6	22.2	12.4	11.1	ns
	Vegetal	13.6	20.8	24.9	20.9	19.3	41.6	26.1	24.9	ns
	Toast bread	15.7^{ab}	36.0^{b}	0.0^{a}	0.0^{a}	11.1^{ab}	0.0^{a}	22.2^{ab}	9.6^{ab}	*
	Apple	38.5^{ab}	13.6^{ab}	13.6^{ab}	52.7^{b}	31.9^{ab}	0.0^{a}	26.1^{ab}	47.1^{b}	*
	Flower	7.9	27.2	23.6	13.6	31.9	20.8	13.6	12.4	ns
	Honey	0.0^{a}	22.2^{b}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	9.6^{ab}	0.0^{a}	*
	Citric	0.0	12.4	9.6	5.6	22.2	20.8	0.0	15.7	ns
Taste	Quality	62.4	63.8	70.1	65.3	70.7	65.3	68.0	70.7	ns
	Sweet	45.8	43.0	44.4	43.0	44.4	25.5	28.9	46.5	ns
	Salty	44.4	38.5	33.3	35.1	41.2	35.1	35.1	36.9	ns
	Acid	65.7	77.0	75.8	79.4	80.5	79.4	80.5	73.3	ns
	Bitter	31.9	49.7	48.1	48.1	43.0	45.8	41.6	51.2	ns
	Body	62.4	63.8	62.4	62.4	62.4	54.4	57.7	60.9	ns
	Persistence	60.9	60.9	63.8	65.3	65.3	65.3	59.3	65.3	ns
	Astringency	41.6	44.4	56.9	62.1	58.3	49.7	51.2	58.3	ns
Global value		59.3	68.0	68.0	68.0	69.4	63.8	69.4	68.0	ns

ns– no significant difference,* – significant difference (p<0.05)

Sensory profiles of wines (only for selected attributes), representing the intensities for aroma and taste as well as global value, are shown in Figure 4.2. Firstly, a comparison was made involving all wines, those produced with free cells (FC) and those produced in consecutive batch fermentations with immobilized cells (B1 to B7). Then, in order to check the evolution of the quality of wines produced in consecutive batch fermentations, a comparison was carried out involving only the two extremes, *i.e.* FC *vs.* B1 and FC *vs.* B7. In general, the profiles of different wines, respecting taste and global value, did not represent obvious differences. However, respecting aroma profiles, some differences could be perceived, particularly when comparing FC *vs.* B1.

A) **B)**

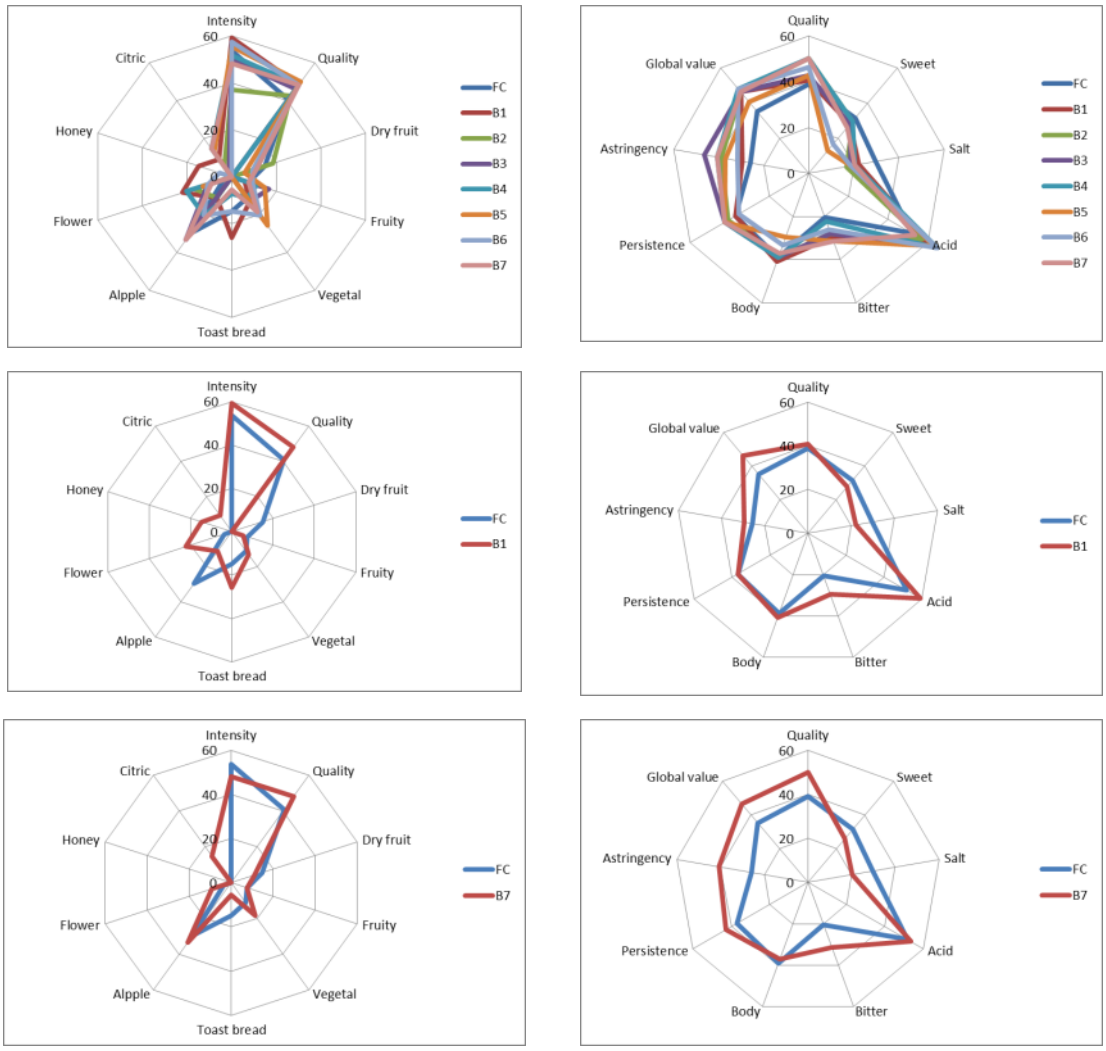

Figure 4.2. Relative intensity (*I*) of sensory descriptors of wines from immobilized and free cells. Aroma profile (**A**) and taste profile and global value (**B**).

The results of triangle test applied to wines produced from free and immobilized cells (FC *vs.* B1 and FC *vs.* B7) are showed in Table 4.8. Considering the number of assessors (22), the minimum number of correct responses required to considered a perceptible difference between the samples (α=0.05) is 12. In our study (Table 4.8), for wine aroma evaluation, only 9 assessors (FC *vs.* B1) or 6

(FC *vs.* B7) correctly identified the samples, representing the 40.0 % and 27.3 % respectively. In the same way, for taste analysis, only 7 (FC *vs.* B1) and 4 (FC *vs.* B7) responses were correct, representing the 31.8 % and 18.2 %, respectively. Although no perceptible differences could be statistically attribute to wines, the panellists are able to better differentiate the pair FC-B1 than the pair FC-B7, indicating greater dissimilarities, between wines produced in the first batch (B1) than those produced in the last batch of the series (B7), when compared to conventional FC wines. On the other hand, the taste analysis was more inconclusive. These results are in agreement with sensory profile of wines performed with the expert panel.

Table 4.8. Triangle test applied to wines produced with free cells (FC) and immobilized cells on batch 1 (B1), and wines produced with free cells (FC) and immobilized cells on batch 7 (B7)

		Total responses	**Correct responses**	
Aroma	FC *vs.* B1	22	9	40.0 %
	FC *vs.*B7	22	6	27.3 %
Taste	FC *vs.*B1	22	7	31.8 %
	FC *vs.*B7	22	4	18.2 %

4.4 Conclusion

Grape skins were found to be an appropriate long-term use support for *S. cerevisiae* immobilization to carry out the alcoholic fermentation in a winemaking process. The immobilized yeasts could be stored at least one month, at 4 °C, without losing its biological activity and operational stability. Furthermore, yeasts were not inhibited by the presence of SO_2 in amounts three times higher than the usual concentration.

After an adaptation period, *i.e.* after three successive batches, immobilized cells on grape skins were able to carry out the complete alcoholic fermentation in 4 d against the 7 d needed with the traditional free cells system. Moreover, the overall quality of the produced wines with both systems became identical.

Finally, the characteristics demonstrated by the proposed immobilized biocatalyst, *i.e. S. cerevisiae* immobilized on grape skins, seem to be easily adaptable to a continuous mode of operation, with the possibility of controlling the winemaking process.

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5. Grape pomace as support for immobilized cell fermentations - evaluation and operational stability.

White wine was produced with *S. cerevisiae* cells immobilized on grape pomace. The support, the main solid waste from the wine industry, consisted of the skins, seeds and stems. Grape pomace was revealed to be an appropriate support for yeast cell immobilization. The wines produced, either with immobilized cells or with free cells, were subjected to chemical analysis by HPLC (ethanol, glycerol, sugars and organic acids) and by gas chromatography (major and minor volatile compounds); additionally, colour (CIELab) and sensory analysis were performed. The use of immobilized systems to conduct alcoholic fermentation in white wine production proved to be a more rapid and a more efficient process, especially when high amounts of SO_2 were present in the must. Furthermore, the final wines obtained with immobilized cells demonstrated improved sensory properties related to the higher amounts of ethanol and volatile compounds produced.

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5.1 Introduction

In the last years, cell immobilization systems has been explored for use in wine production, to conduct alcoholic fermentation (Kourkoutas *et al*, 2005; Mallouchos *et al*, 2003; Tsakiris *et al*, 2004) , as well as in malolactic fermentation (Agouridis *et al*, 2008; Durieux *et al*, 2000; Maicas *et al*, 2001). Achievements in this area of research are very important as there are attempts to reduce the operating costs, to control the fermentation processes and to increase the quality of the final product – the wine.

Cell immobilization systems utilized for alcoholic fermentations have technological and economic advantages when compared with free cell systems, such as increased productivity, higher cell concentrations in the reactors, possible reuse of the biomass in consecutive batch processes, greater tolerance of the cells to inhibitory substances and the possibility of operating the processes in a continuous mode (Cachon and Diviès, 2001; Junter and Jouenne, 2004; Kourkoutas *et al*, 2004; Genisheva *et al*, 2011). The immobilization techniques can be divided into four categories: attachment to a support, entrapment in a porous matrix, cell aggregation and containment behind a barrier (Pilkington *et al*, 1998; Kourkoutas *et al*, 2004; Verbelen *et al*, 2006).

The supports to be used in the alcoholic beverage industry should have high resistance and stability, should not damage the quality of the final product and have food-grade purity (Kourkoutas *et al*, 2004; Verbelen *et al*, 2006). Some inorganic supports such as the mineral *kissiris* (Bakoyianis *et al*, 1992) and *γ*-alumina (Loukatos *et al*, 2000), have been used successfully for the immobilization of *Saccharomyces cerevisiae*. However, some of these supports may be undesirable, due to the release of mineral residues into the final product (Loukatos *et al*, 2000). Organic supports, mainly of natural origins, such as pieces of fruit, are a good alternative, where the cells adhere to the surface by natural adsorption. Apple (Kourkoutas *et al*, 2002b), quince (Kourkoutas *et al*, 2002a), pear (Mallios *et al*, 2004), watermelon (Reddy *et al*., 2008), grape skins (Mallouchos *et al*, 2002) and dried raisin berries (Tsakiris *et al*, 2004) have already been studied and have advantages on an industrial scale, as they are of food grade purity and could reduce the cost of the process.

Grape pomace is the most plentiful solid waste of the wine industry. It results from the pressing of grapes and consists mostly of skins, seeds and stems. Traditionally, it is used to produce spirits or as fertilizer. It is also utilized to obtain value-added products (Lu and Foo, 1999; Amico *et al*, 2004), such as enzymes (Botella *et al*, 2005), extracts with antibacterial activity (Özkan *et al*, 2004), grape seed oil, anthocyanic dyes and tartaric acid (Bourseaux *et al*., 1998). As this is a by-product that is always extensively generated in wine production, it is important to find alternative uses.

From a consumer point of view, flavour is one of the most valuable attributes contributing to the overall quality of a wine. Aroma volatile compounds are the primary contributors to wine flavour, producing an effect on the sensory senses of the taster (Vilanova *et al*., 2010). Colour is another

parameter connected to the quality of the wine. It gives an idea of the evolution of the wine in time and of the existence of possible defects (Almela *et al*., 1995). One valuable technique for distinguishing between wines is sensory evaluation. Sensory tests can discriminate between wines and estimate the quality of wine produced using different oenological practices (Tsakiris *et al*., 2006).

The aim of the present study was to produce white wine, with *S. cerevisiae* immobilized on grape pomace by natural adsorption, and to compare this wine to wines produced using free cells. Sensory characteristics colour and volatile aroma compounds were evaluated.

5.2 Materials and Methods

5.2.1 Inoculum preparation

A commercial *Saccharomyces cerevisiae* strain (Lalvin QA23, Proenol) was used in the experiments. The inoculum was prepared by cultivation of the yeast in 500 mL Erlenmeyer flasks containing 200 mL of YPD medium with the following composition (g/L): yeast extract (10), peptone (20) and glucose (20). Cells were cultivated under static conditions, at 30 ^{o}C for 24 h, being subsequently recovered by centrifugation (*RCF*=7000, 20 min), washed with distilled water and re-suspended in the fermentation medium to obtain an initial concentration of 1 g/L (dry weight).

5.2.2 Support materials for cell immobilization

Grape pomace, constituted by stems, seeds and skins, picked randomly after crushing and pressing of indistinct white grapes, was used as support material for cell immobilization. This support material was supplied by a local winemaking company, being washed with distilled water and dried at 60 ºC, until constant weight, before use.

5.2.3 Immobilization of cells

S. cerevisiae cells (1 g/L; dry weight) and 2 g of dry grape pomace, previously sterilized at 121°C for 20 min, were added to 200 mL of a complex culture medium composed of (g/L): glucose (120), yeast extract (4), $(NH_4)_2SO_4$ (1), KH_2PO_4 (1) and $MgSO_4$ (5). The mixture was left to ferment in 500 mL Erlenmeyer flasks under static conditions at 30 °C for 24 h (Figure 5.1). To compare the effect of the medium composition on immobilization efficiency the same procedure was performed in 200 mL of diluted grape must (≈ 120 g/L of total sugars, glucose and fructose).

The final immobilization experiments, carried out to produce white wine, were performed in 300 mL of diluted grape must (≈ 120 g/L of total sugars) and in 300 mL of raw grape must (≈ 210 g/L of total sugars) with 1 g/L of *S. cerevisiae* cells (dry weight). In each broth, 50 g of dry sterilized grape pomace was added for cell immobilization at 25 °C for 78 h, with agitation (200 min^{-1}). The

biocatalyst prepared in raw grape must was washed twice with grape must and reused for the subsequent batch fermentations.

5.2.4 Fermentation conditions

The alcoholic fermentations for the winemaking process were performed in two different series, each one including two consecutive batches with immobilized cells (batch 1 and batch 2) as depicted in Figure 5.2. In the first series (series 1), 60 g of wet grape pomace with immobilized cells (corresponding to 0.75 g of dry weight of cells) was placed in 3 L of grape must, *i.e.* a cell concentration of 0.25 g/L. The density was monitored daily and the fermentation was stopped when it reached 0.995 g/mL. After that, the support was recovered and washed twice with grape must and reused for the second batch fermentation. Free cell fermentations, with the same cell concentration, were performed as controls. In the second fermentation series (series 2), the procedure was the same, but 400 g of wet grape pomace with immobilized cells was used (corresponding to 5.78 g dry weight of cells) in 2.75 L of grape must, *i.e.* a cell concentration of 2.10 g/L. All experiments were performed at room temperature (≈ 22 °C), without agitation and in duplicate.

After the addition of sulfur dioxide (30 mg/L) and bentonite (600 mg/L), the produced wines were stabilized at 4 °C over 15 d. Then, they were filtered and the SO_2 concentration was again adjusted to 30 mg/L before bottling. HPLC, gas-chromatographic and colour, as well as immobilized cells concentration determinations were performed on the finished wines. Sensory analysis was performed only for wines from the 2nd series.

5.2.5 HPLC analysis

Glucose, fructose, ethanol, glycerol and organic acids (citric, tartaric, malic, succinic lactic and acetic) concentrations were determined by high performance liquid chromatography (HPLC) in a Jasco chromatograph equipped with a refractive index detector (Jasco 830-RI), an ultraviolet detector and a Varian Metacarb 67H column (300 mm × 6.5 mm) operated at 80 ºC. A 5 mmol/L H_2SO_4 solution was used as eluent at a constant flow rate of 0.3 mL/min. Identification of metabolites was performed by comparing retention times with those of pure standard compounds and quantification was carried out after external standard calibration.

5.2.6 Gas-Chromatographic analysis

Major volatile compounds were directly analysed after adding 292.5 μg of 4-nonanol (internal standard – IS) to 5 mL of wine. Minor volatile compounds were analysed after extraction of 8 mL of wine with 400 μL of dichloromethane, spiked with 2.91 μg of 4-nonanol (IS), according to the methodology proposed by Oliveira *et al.* (2006). All analyses of volatiles were carried out in triplicate.

A Chrompack CP-9000 gas chromatograph equipped with a split/splitless injector and a flame ionization detector (FID) with a capillary column, coated with CP-Wax 52 CB (50 m × 0.25 mm; 0.2 μm film thickness, Chrompack), was used. The temperatures of the injector and the detector were both set to 250 °C. The oven temperature was held at 60 °C, for 5 min, then programmed to rise from 60 °C to 220 °C, at 3 °C/ min, and held at 220 °C for 10 min. The carrier gas was helium 4× (Praxair) at 120 kPa. Major volatile compounds were analysed in split mode (13 mL/min) injecting 1 μL of sample, and the extracts containing minor volatile compounds were injected – 3 μL – in splitless mode (for 15 s).

Identification of volatiles was performed with Varian MS Workstation software, version 6.6, by comparing retention indices with those of pure standard compounds. Minor volatile compounds were quantified as 4-nonanol equivalents only.

5.2.7 Colour analysis

The colour of the wines was assayed by the CIELab method, by measuring the absorbance between 380 nm and 770 nm (data pitch = 2 nm), using a Jasco UV/Vis V-560 spectrophotometer. The recorded data were processed by an algorithm using the program Matlab version r2010a, developed by the Science of Vision and Colour Laboratory, Department of Physics, University of Minho, to obtain the CIELab coordinates, L^*, a^* and b^*. These coordinates allowed the determination of other three parameters in the produced wines: saturation (C^*), variation in saturation (ΔC^*) and variation in lightness (ΔL^*), according to Almela *et al*, 1995. The following equations were used:

$$C^* = \sqrt{a^{*2} + b^{*2}} \qquad \text{(Equation 1)}$$

$$\Delta C^* = C^*_{\mathrm{x}} - \bar{C}^* \qquad \text{(Equation 2)}$$

$$\Delta L^* = L^*_{\mathrm{x}} - \bar{L}^* \qquad \text{(Equation 3)}$$

C_x^* and L_x^* are the saturation and lightness of the wines produced by immobilized cells, and $\bar{C}^*$ and $\bar{L}^*$ are the saturation and lightness, respectively of the reference wines, *i.e*. wines produced with free cells.

5.2.8 Sensory analysis

The three wines produced in the second series of fermentations were subjected to sensory analysis, in dark glasses, using a triangular test (Norm ISO 4120, 2004). Six sets of three glasses were prepared, of which two contained the same wine (Table 5.1). The glasses were identified on the basis of random numbers with three digits and contained 30 mL of wine. The tests were conducted using 35 panellists without significant experience, at the Laboratory of Food Science and Technology, Centre of

Biological Engineering, University of Minho. The panellists were also asked to name a preference in each of the series of the three wines.

Table 5.1. Composition of each set of three glasses used in the sensory evaluation of the 2nd series of fermentations

Set	Wines		
	Glass 1	**Glass 2**	**Glass 3**
1	free cells	free cells	batch1
2	free cells	free cells	batch2
3	batch1	batch1	batch2
4	batch1	batch1	free cells
5	batch2	batch2	free cells
6	batch2	batch2	batch1

5.2.9 Immobilized cell determination

The immobilized cell concentration was determined at the fermentation's end by washing the biocatalyst with 30 g/L NaOH solution, for 24 h, at 30 ºC and an agitation rate of 120 min^{-1}, according to Genisheva *et al.*, (2011). The free cell concentration in the fermentation medium was estimated by measuring the absorbance at 600 nm, which was correlated to a calibration curve (dry weight *vs* absorbance).

5.2.10 Statistical analysis

All of the fermentation experiments were conducted in duplicate. The results were analysed by ANOVA, using FAUANL software (Olivares, 1994). Tukey´s test was used to detect significant differences between samples.

5.3 Results and Discussion

The ability of immobilized *S. cerevisiae* to ferment grape must was evaluated by measuring glucose and fructose consumption, ethanol, glycerol, major volatile and minor volatile compound production, sensory evaluation and chromatic characteristics.

5.3.1 Immobilization of Saccharomyces cerevisiae

The immobilization of the yeast cells was performed in three different immobilization media: complex culture medium, diluted grape must and raw grape must (Figure 5.1). Initially, a comparison was performed between fermentations with two different media: complex culture and diluted grape must. The quantities of the immobilized cells per mass of support, X_{im}, at the end of immobilization runs in complex medium and in diluted must were 14.90 mg/g and 16.10 mg/g, respectively; these

results showed no significant differences (p<0.05). Nevertheless the two assays showed a significant difference (p<0.05) in terms of the free biomass produced. The free cell concentrations in the complex culture medium and diluted grape must were 6.35 g/L and 4.80 g/L, respectively. Therefore, the assays with the complex culture medium had a higher total concentration of cells than the assays with the diluted must, but showed a lower immobilization efficiency (data not showed).

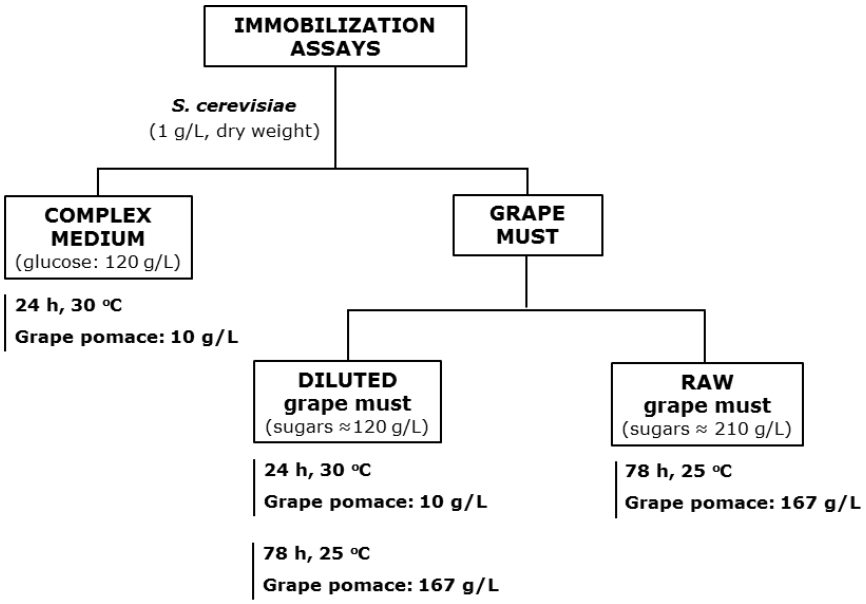

Figure 5.1. Flow chart for the immobilization assays.

The composition of the diluted must may have favoured the stability of cells on the support, and even influenced the yeast's own metabolism, since it was rich in sugars, acids, amino acids, minerals and pectic substances, amongst others, some of which were absent in the complex medium. During the immobilization process in the complex medium, 127 g/L of initial glucose was almost completely consumed after 16 h (residual glucose was 5.5 g/L). In relation to immobilization in diluted must, the total initial concentration of sugars was 132 g/L (53 g/L glucose and 79 g/L of fructose) and after 16 h there were still 52.7 g/L of sugars remaining (15.3 g/L glucose and 37.4 g/L of fructose). This suggests that the yeast took longer to adapt to the environment and, therefore, to take up these sugars.

According to the previous results and with the purpose of producing larger amounts of immobilized support for further use in fermentations, immobilization runs were carried out using 50 g of support material and diluted or raw grape must (Figure 5.1). As the initial total sugar concentration of the raw must was 210 g/L, the immobilization was conducted for a longer period (78 h). The amount of immobilized cells was measured throughout the process, as shown in Figure 5.2.

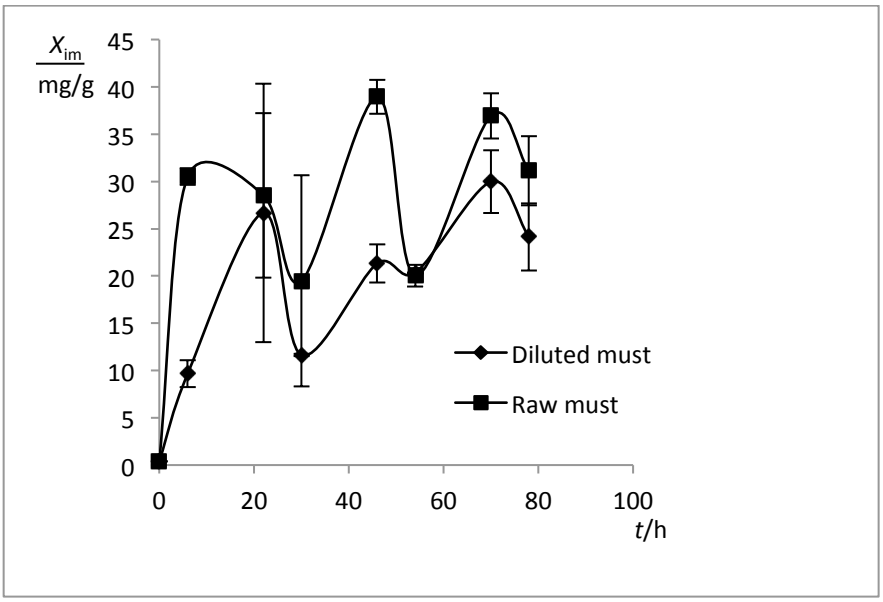

Figure 5.2. Variation of the mass of the immobilized cells per gram of support (X_{im}) over time (t) in raw and diluted grape must.

The concentration of immobilized yeast cells in the two musts varied greatly over the 78 h and was higher in raw grape must. The highest amount detected of immobilized cells per mass of support of 40 mg/g was after the 46th hour. The immobilizations with a high amount of support were carried out under agitation, unlike previous tests, to ensure that the support was always immersed in the medium, thus allowing maximum contact between the immobilized cells and the medium constituents. The media agitation during the immobilization process and the absence of barriers between cells and the medium, possibly favoured the constant desorption and replacement of microorganisms in the media. The agitation may have had a negative effect on the stability of the biofilm (Genisheva *et al*., 2011). However, it was necessary in order to facilitate contact between the cells and the support and to promote a more homogeneous distribution of the constituents of the must. Note that the tests were performed with only enough juice to involve the support material, in order to reduce the amount of spent must. The raw grape must appeared to be the best option for immobilizing the yeast cells, since it allowed for the immobilization of more cells, as well as prior adaptation of their metabolism to the fermentation medium.

5.3.2 Fermentation trials with immobilized cells

S. cerevisiae cells, previously immobilized on grape pomace, were used for the fermentation of grape must. For comparison, fermentations under the same conditions but without the addition of the support were performed (Figure 5.3). Two series of fermentation were conducted, each with two repeated batch runs (1 and 2). Batch 1 was carried out with the previously immobilized grape pomace, which was separated from the liquid at the end of the alcoholic fermentation, washed with grape must

and reused in the batch 2 run. The two series were performed with different quantities of biocatalyst, 60 g and 400 g (wet weight), respectively.

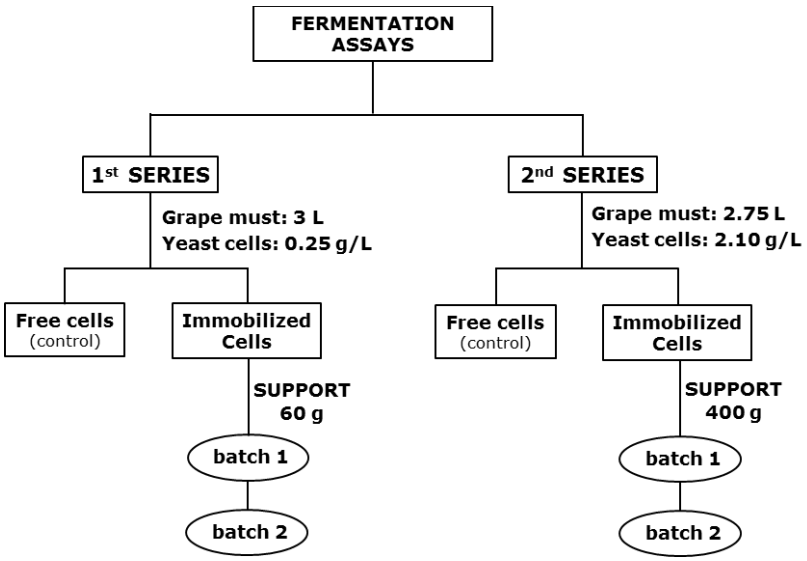

Figure 5.3. Flow chart for the fermentation assays.

The provided must had a high concentration of free SO_2 (54.4 mg/L). Therefore, the first series of fermentations were conducted under an inadequate environment for yeast development, causing a delay at the beginning of the process. The experiments with the free cells did not start until almost half of the SO_2 was removed and there was a supplementary addition of 0.5 g/L of the yeast cell suspension. However, the high concentration of free SO_2 did not appear to exert a negative influence over the fermentation assays with the immobilized yeast cells. This suggested that the immobilized yeast cells were more tolerant to the high quantities of free SO_2. This is important since SO_2 is one of the main inhibitors of yeast cell growth, thus helping in the conservation of the must for longer time periods (Ribéreau-Gayon *et al*., 2006). Wine producers usually encounter problems when trying to ferment grape musts with high concentrations of SO_2. The use of immobilized cells may be a solution to this problem.

Free cell fermentations of the first series were complete on the 22nd day, while fermentations with the immobilized cells, batch 1 and batch 2, were complete on the 14th day and the 11th day, respectively. Regarding the second series, fermentation runs with free cells were more rapid (4 d) than the two consecutive batches with immobilized cells (6 d and 7 d, respectively). This was probably due to substrate diffusion problems in the fermentation flask which was full of the immobilized support. As a result of these diffusion problems, series 2 presented lower amounts of immobilized cells for batch 1 and batch 2. The immobilized biomass was 70.45 mg/g and 62.61 mg/g, respectively; in contrast to the first series where the concentrations for batch 1 and batch 2 were 106.05 mg/g and 111.92 mg/g respectively.

Since some cells would always be floating off the immobilization media, the fermentations were probably carried out by a mix of free and immobilized cells. Moreover, the support material used for immobilization – grape pomace – is not inert, and may affect the success and stability of the fermentation runs because yeasts could metabolize some constituents and, concomitantly, colour compounds may be released to the wine. Nevertheless, the treatment carried out before immobilization and also the immobilization procedure itself certainly soften these effects.

5.3.3 HPLC analysis

The concentrations of glucose, fructose, glycerol, ethanol and organic acids (citric, tartaric, malic, succinic, lactic and acetic) determined by HPLC can be seen in Table 5.2. The residual sugars concentration was low in all of the wines, varying between 0.10 g/L and 0.54 g/L for glucose and between 1.08 g/L and 8.76 g/L for fructose. In the fermentations with free cells, the glycerol concentration was higher than in fermentations with immobilized cells. Nevertheless the levels were in the usual range, *i.e.* 5 g/L to 15 g/L (Ribéreau-Gayon *et al*., 2006). With regard to ethanol, only the fermentations with immobilized cells in the batch 2 of the 2nd series showed higher concentrations compared to the fermentations with free cells.

Table 5.2. Mean concentrations (*C*) of sugars, organic acids, ethanol and glycerol analysed by HPLC at the end of the alcoholic fermentation

Compound	*C*/(g/L)					
	1st series *			**2nd series** **		
	free	**batch 1**	**batch 2**	**free**	**batch 1**	**batch 2**
glucose	0.26^{bc}	0.38^{ab}	0.41^{ab}	0.10^{c}	0.30^{b}	0.54^{a}
fructose	5.37^{b}	4.96^{b}	5.45^{b}	1.08^{d}	2.49^{c}	8.76^{a}
glycerol	5.73^{bc}	4.47^{c}	4.59^{c}	7.20^{a}	4.69^{c}	6.61^{ab}
ethanol	77.06^{ab}	79.21^{ab}	80.63^{ab}	64.13^{b}	70.83^{b}	95.42^{a}
citric acid	0.25^{a}	0.43^{a}	0.41^{a}	0.36^{a}	0.54^{a}	0.42^{a}
tartaric acid	2.74^{bc}	3.20^{abc}	3.56^{ab}	2.32^{c}	2.37^{c}	3.93^{a}
malic acid	4.58^{a}	4.47^{a}	4.79^{a}	2.95^{a}	3.68^{a}	4.38^{a}
succinic acid	2.33^{a}	2.22^{a}	2.26^{a}	2.51^{a}	2.63^{a}	3.19^{a}
lactic acid	2.07^{c}	1.84^{c}	1.97^{c}	3.03^{b}	2.81^{b}	3.69^{a}
acetic acid	0.51^{ab}	0.29^{ab}	0.33^{ab}	0.58^{a}	0.16^{b}	0.49^{ab}

a, b, c, d – values with the same letters mean no significant difference at 95 % confidence level between fermentation assays; * – cell concentration of 0.25 g/L; ** – cell concentration of 2.10 g/L

Since immobilized cells are more tolerant to inhibitors (Norton and D'Amore, 1994), they could maintain their fermentation activity even when the alcohol content was high. Ethanol affects the metabolic activity of yeasts, influencing the type and amount of volatile compounds produced and also acts as a substrate for the formation of several ethyl esters (Jackson, 2008).

In all of the wines produced, the tartaric, malic and succinic acid concentrations were the highest of the six acids analysed. Malic and tartaric acids are normally found in high amounts in grapes and musts, and do not undergo large changes during fermentation, while succinic acid is a by-product of the metabolism of yeasts (Ribéreau-Gayon *et al*., 2006), which may explain the recorded values (between 2.22 g/L and 3.19 g/L). Citric acid is usually present at very low concentrations in wines (Costantini *et al.*, 2009) and this was also true in the present study. The lactic acid concentration was similar in all of the fermentations. Acetic acid was found in higher concentrations in the free cell fermentations than in the fermentations with the immobilized cells. Nevertheless the values were always below the legal limit for white wines of 1.2 g/L (OIV, 2012a).

5.3.4 Major volatile compounds

The concentrations attained for the major volatile compounds, identified and quantified by GC-FID, are shown in Table 5.3. This group includes acetaldehyde, ethyl acetate, methanol and the higher alcohols (1-propanol, 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol).

Acetaldehyde reached concentrations up to 8.6 mg/L, values lower than its orthonasal perception threshold of 10 mg/L (Moreno *et al*., 2005). Although fermentations conducted by free cells for both series presented similar results, fermentations with immobilized cells in the first series (batch 1 and batch 2) produced higher amounts of acetaldehyde; for the second series, immobilized and free systems presented similar results. Usually, acetaldehyde is present in concentrations below 75 mg/L in young wines (Hornsey, 2007). Kourkoutas *et al*., (2002a; 2006) however, found amounts of 13 mg/L to 106 mg/L in wines produced with immobilized cells on quince and apple. This compound can confer fresh, green and even oxidized notes to wines (Czerny *et al*., 2008; Hornsey, 2007).

Ethyl acetate has a perception threshold of 7.5 mg/L (Guth *et al*., 1997, Moreno *et al*., 2005), contributing to the "fruity" and "solvent" character of wines (Sánchez-Palomo *et al*., 2007). It was found in higher concentrations in fermentations with immobilized cells. Moreover, batch 2 presented higher amounts of ethyl acetate than batch 1, a fact observed for both series of fermentations. Batch 2 of series 2 achieved the highest levels for this compound. In all of the fermentations, this compound was found in concentrations higher than its perception threshold.

In regard to methanol, all fermentations presented lower levels (17.0 mg/L to 33.4 mg/L) than what has been published for Turkish white wines, 30.5 mg/L to 121.4 mg/L (Cabaroglu, 2005). Wines

produced with cells immobilized on quince fruit have a reported methanol content of under 100 mg/L (Kourkoutas *et al*., 2002). The low concentration of methanol found in the wines produced in these experiments is a positive finding. Methanol concentration is under regulatory control due to its toxic nature and the permitted limit, according to OIV (2012b), is 250 mg/L. Methanol results from the pectin of the skin of the grapes which undergoes an enzymatic conversion (Ribéreau-Gayon *et al*., 2006). Since the fermenting must was in contact with the grape skins for a long time period, this could lead to elevated amounts of methanol in the product. However, this study has shown only low amounts of methanol to be present, even when a higher amount of grape pomace was used as the support in the immobilized system (series 2).

From the identified higher alcohols, 3-methyl-1-butanol showed the highest concentration (between 113.9 mg/L and 193.6 mg/L), and was above its perception threshold of 30 mg/L (Guth *et al*., 1997). This alcohol may contribute to the "sweet" and "fusel" odour descriptors of wines (Gómez-Míguez *et al*., 2007). Although higher alcohols, individually, do not give pleasant notes to the wine (except 2-phenylethanol), together they can contribute positively to the overall aroma. Some authors have stated that 300 mg/L is the limit for a positive contribution (Rapp and Versini, 1995). Higher concentrations can bring strong and pungent notes to the wine (Nykänen, 1986). Nevertheless, the particular impact of each volatile component or group of components, to the overall aroma of wine depends on its composition and on the concentration and the perception thresholds (Tsakiris *et al*., 2006). Only batch 1 of the second fermentation series presented more than 300 mg/L for the sum of the higher alcohols. Comparable results were observed by Kourkoutas *et al.*, (2006) when batch fermentations were conducted at low temperatures with immobilized *S. cerevisiae*. An interesting higher alcohol was 2-phenylethanol, which presented concentrations between 19.5 mg/L and 34.9 mg/L, always above the perception threshold of 10 mg/L, thus giving "rose" and "sweetish" nuances to the wine (Escudero *et al.,* 2004; Guth *et al*., 1997). Oliveira *et al*. (2008) reported similar concentrations for this compound in *Loureiro* (31.3 mg/L) and *Alvarinho* (21 mg/L) wines. The levels of 2-phenylethanol in wine are mainly related to the amino acids metabolism of the yeast during the fermentation (Henschke and Jiranek, 1993).

Table 5.3. Mean concentrations (*C*) and confidence limits ($p = 0.05$), of the major volatile compounds at the end of alcoholic fermentation

Compound	1st series *						2nd series **					
	free cells		batch 1		batch 2		free cells		batch 1		batch 2	
	C/(mg/L)	±	*C* /(mg/L)	±	*C* /(mg/L)	±	*C*/(mg/L)	±	*C*/(mg/L)	±	*C*/(mg/L)	±
acetaldehyde	$2.6^{b,c}$	1.7	8.6^{a}	5.6	5.1^{b}	3.3	$2.6^{b,c}$	2.2	1.4^{c}	0.4	$2.4^{b,c}$	2.1
ethyl acetate	$28.3^{c,d}$	0.9	30.2^{c}	3.0	37.7^{b}	2.5	23.2^{d}	5.8	$27.9^{c,d}$	3.1	50.4^{a}	8.3
methanol	18.1^{b}	6.5	20.7^{b}	6.6	16.9^{b}	1.7	17.0^{b}	4.8	32.8^{a}	6.5	33.4^{a}	4.2
1-propanol	16.2^{c}	5.0	$18.0^{b,c}$	1.4	$20.2^{b,c}$	4.2	16.1^{c}	1.1	21.9^{b}	0.9	41.5^{a}	6.9
2-methyl-1-propanol	21.2^{d}	4.4	36.1^{b}	5.8	37.9^{b}	4.1	$25.8^{c,d}$	3.2	29.9^{c}	1.6	44.5^{a}	7.0
2-methyl-1-butanol	31.1^{a}	5.7	$26.8^{a,b}$	4.0	$21.8^{b,c}$	3.7	$26.6^{a,b}$	2.8	32.8^{a}	2.2	18.4^{c}	12.8
3-methyl-1-butanol	162.3^{b}	16.9	163.4^{b}	18.6	$133.0^{c,d}$	10.0	113.9^{d}	8.6	193.6^{a}	7.2	$144.5^{b,c}$	44.6
2-phenylethanol	34.9^{a}	13.7	$26.3^{a,b,c}$	4.7	19.5^{c}	4.1	$23.1^{b,c}$	7.1	$33.1^{a,b}$	15.4	$25.5^{a,b,c}$	2.3

a, b, c, d – values with the same letters mean no significant difference at 95 % confidence level between fermentation assays

* – cell concentration of 0.25 g/L; ** – cell concentration of 2.10 g/L

5.3.5 Minor volatile compounds

In total, 14 minor volatile compounds were identified and quantified, including 7 esters, 3 alcohols, 3 volatile fatty acids and 1 C_{13}-norisoprenoid. The respective concentrations and level of significance (p<0.05) are shown in Table 5.4. In general, fermentations with immobilized cells had higher amounts of the minor volatile compounds, with the resulting wines having a more pleasant and rich aroma profile. Moreover, the concentration increased from batch 1 to batch 2 (Table5.4).

The statistical analysis regarding concentrations of the minor volatile compounds showed no difference between the fermentations with reference to the five following compounds: ethyl lactate, hexan-1-ol, *E*-3-hexen-1-ol, *Z*-3-hexen-1-ol and β-damascenone. However, all the concentrations found for β-damascenone were above the perception threshold of 0.05 μg/L (Guth *et al*., 1997), thus bringing "sweet", "apple" and "dry plum" nuances to the wines (Escudero *et al*., 2004; López *et al*., 2004). For the other nine analysed compounds, significant differences were found (p<0.05). The free cell fermentations were found to differ from the immobilized cell fermentations regarding the following aromatic compounds: ethyl butyrate, isoamyl acetate, ethyl hexanoate, hexyl acetate, ethyl octanoate, 2-phenylethyl acetate, hexanoic acid, octanoic acid and decanoic acid. Ethyl butyrate and octanoic acid, bring "fruity" and "fatty" characteristic (Escudero *et al*., 2004), respectively, and were present in all of the wines in concentrations above their perception thresholds of 20 μg/L and 500 μg/L respectively (Ferreira *et al*., 2000; Moreno *et al*., 2005). For isoamyl acetate (3-methylbutyl acetate), no differences were observed for the free cell fermentations (both series), but in contrast to the previous observations the assays with immobilized cells were different between each other. Isoamyl acetate was found in concentrations higher than its perception threshold, 30 μg/L (Guth *et al*., 1997; Moreno *et al*., 2005) for all fermentations assays, bringing "banana" descriptors to the overall aroma of wine (Escudero *et al*., 2004). Similarly, concentrations of ethyl octanoate with free cells (both series) were different from each other and from the immobilized cells. However the assays with immobilized cells were equal to each other. All the wines produced had concentrations of ethyl octanoate above the perception threshold of 5 μg/L (Ferreira *et al*., 2000), bringing "fruity" and "fresh" notes to wines (Escudero *et al*., 2004). The concentrations of ethyl hexanoate (fruity and flowery notes according to Escudero *et al.*, (2004) and López *et al.* (2004)) were found to be different for all the fermentations (p<0.05); moreover, with immobilized cells, the obtained concentrations were above the perception threshold of 5 μg/L (Moreno *et al*., 2005). The concentrations for 2-phenylethyl acetate in batch 2 from both series were similar (batch 2, series 1) or even above (batch 2, series 2) the perception threshold of 250 μg/L (Guth *et al*., 1997, Moreno *et al*., 2005) thus contributing with "flowery" notes (López *et al*., 2004). Decanoic acid (fatty), in wine produced in batch 2 from series 1, was found in concentration above the perception threshold of 1000 μg/L (Ferreira *et al*., 2000).

Table 5.4. Mean concentrations (*C*) and confidence limits ($p = 0.05$) of the minor volatile compounds at the end of alcoholic fermentation

Compound	1st series *						2nd series **					
	free cells		batch 1		batch 2		free cells		batch 1		batch 2	
	C/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±	*C*/(μg/L)	±
ethyl butyrate	35.8[a]	54.8	91.5[b]	35.7	119.4[b]	92.0	32.8[a]	46.7	129.4[b]	10.8	120.9[b]	9.5
isoamyl acetate	140.1[d]	199.3	621.0[c]	424.3	1032.4[b]	523.8	281.0[d]	27.3	1035.2[b]	78.2	1596.3[a]	162.6
ethyl hexanoate	85.2[e]	98.5	290.2[c]	99.2	327.9[b,c]	23.0	166.6[d]	19.6	548.8[a]	9.8	360.0[b]	17.4
hexyl acetate	1.7[c]	3.8	25.4[a,b]	42.2	33.2[a]	4.8	6.1[b,c]	0.4	27.9[a]	1.8	39.0[a]	4.7
ethyl lactate	336.7[a]	473.0	343.4[a]	345.6	224.9[a]	340.6	150.2[a]	33.5	131.6[a]	33.0	279.2[a]	42.1
hexan-1-ol	314.8[a]	388.6	240.0[a]	160.9	212.6[a]	189.3	189.3[a]	24.9	182.3[a]	23.6	246.3[a]	35.0
E-3-hexen-1-ol	5.1[a]	7.2	10.1[a]	10.0	9.9[a]	9.8	7.4[a]	6.4	6.8[a]	0.9	10.6[a]	0.9
Z-3-hexen-1-ol	7.0[a]	5.5	8.2[a]	4.7	10.0[a]	8.2	8.0[a]	1.4	7.6[a]	5.6	7.2[a]	4.1
ethyl octanoate	15.5[c]	11.6	168.8[a]	27.0	207.2[a]	81.1	79.1[b]	7.2	190.2[a]	6.5	191.8[a]	18.7
2-phenylethyl acetate	166.5[c]	93.6	173.2[c]	80.0	247.6[b]	79.4	80.3[d]	5.4	209.0[b,c]	11.0	434.6[a]	3.4
β-damascenone	1.7[a]	7.3	1.6[a]	3.7	4.2[a]	3.8	3.3[a]	0.3	2.8[a]	5.9	1.4[a]	0.7
hexanoic acid	871.2[a,b]	768.0	944.5[a,b]	585.9	727.6[a,b]	389.6	563.6[b]	46.3	1079.9[a]	216.7	722.4[a,b]	70.2
octanoic acid	1658.4[d]	877.5	4104.9[a]	1021.4	3938.5[a]	1323.5	2367.3[c,d]	178.8	3247.3[a,b]	150.3	2845.6[b,c]	80.0
decanoic acid	173.3[b]	175.7	793.3[a,b]	490.3	1184.8[a]	834.6	360.9[a]	40.9	364.8[b]	24.1	528.8[b]	48.1
TOTAL	**3530.0**		**7816.1**		**8280.2**		**4295.9**		**7163.6**		**7384.1**	

a, b, c, d – values with the same letters mean no significant difference at 95 % confidence level between fermentation assays

* – cell concentration of 0.25 g/L; ** – cell concentration of 2.10 g/L

5.3.6 Colour analysis

The colour of the wine is another important characteristic from the consumer´s point of view. For this reason, colour analysis of the wines was carried out using the CIELab method, with the determined coordinates L^*, a^* and b^*. Furthermore, in order to compare the wines, variation in saturation, ΔC^*, and variation in lightness, ΔL^*, were also calculated (Table 5.5).

Table 5.5. CIELab coordinates and the calculated values for C^*, ΔL^* and ΔC^*

Parameter	1st series						2nd series					
	free cells		batch 1		batch 2		free cells		batch 1		batch 2	
		±		±		±		±		±		±
L^*	$95.92^{a,b}$	0.03	$95.64^{b,c}$	0.24	$95.96^{a,b}$	0.34	96.18^{a}	0.21	94.94^{d}	0.33	95.43^{c}	0.73
a^*	-0.43^{d}	0.01	-0.62^{c}	0.02	-0.40^{d}	0.01	-0.45^{d}	0.04	-1.48^{a}	0.02	-1.06^{b}	0.14
b^*	$0.03^{d,e}$	0.07	0.11^{c}	0.28	0.12^{d}	0.30	0.07^{e}	0.18	0.06^{b}	0.15	0.27^{a}	0.66
C^*	2.03	0.06	2.84	0.28	2.06	0.29	1.69	0.17	6.63	0.14	4.99	0.62
ΔL^*	0.00		-0.28		0.03		0.00		-1.24		-0.75	
ΔC^*	0.00		0.81		0.03		0.00		4.94		3.29	

a, b, c, d – values with the same letters mean no significant difference at 95 % confidence level-, $L,^* a^*, b^*$ – CIELab coordinates, C^* – saturation of colour, ΔC^* – variation of saturation, ΔL^* – variation of lightness

The results obtained for the coordinates L^*, a^* and b^* showed significant differences between wines in terms of the colour parameters (test of Tukey). Exceptions were the fermentations with the free cells and batch 2 from series 1 (Table 5.5). These two wines were alike in terms of the colour parameters. Since it was found that the wines were indeed different in respect to the parameters L^*, a^*, b^*, the average values for each parameter were compared. Wines produced with a greater amount of support (batch 1 and batch 2, series 2) presented the lower values of L^* (lower brightness and higher opacity), which suggests that these wines have a higher colour intensity. These results suggest that increasing the amount of support used in the fermentation process directly influenced the intensity of the colour of the wines. The parameter C^* was higher for the wines in batch 1 and batch 2 (series 2), indicating a higher colour vividness. All the wines showed the coordinate values a^* below zero and the coordinate b^* greater than zero, indicating a shift towards the green and yellow colour, respectively.

The wines from batch 1 with immobilized cells from both series, had a higher colour intensity (lower values of L^*) as well as increased colour saturation (higher values of C^*), compared to the wines produced with immobilized cells in batch 2. Figure 5.4 shows the differences in colour of

the produced wines, using a graphical representation of ΔL^* as function of ΔC^*, which reduces the CIELab coordinates into a two-dimensional colour space (Almela *et al*., 1995). Thus, the deviations in the colour of the wines produced by the immobilized cells, compared to those produced with free cells, could be observed. It was found that the wines produced in the first fermentation (batch 1) with immobilized cells had a darker colour than those produced in the second fermentation (batch 2), due to the lower values of ΔL^*. This fact could be attributed to the release of some coloured compounds from the grape pomace; however, as the number of batches increased, the colour tended to stabilize.

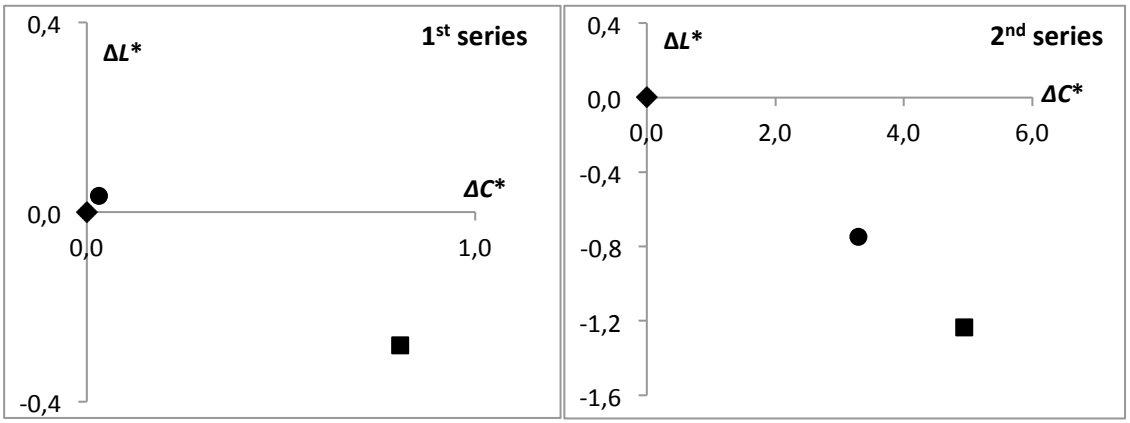

Figure 5.4. Variation of saturation ΔC^*, and variation of lightness, ΔL^*, of wines produced in both fermentation series (♦ free cells, ■ batch 1, • batch 2).

5.3.7 Sensory analysis

A triangular test was used to evaluate possible differences between the two products, based on the analysis of three samples, in which the taster had to decide which one of the three samples was different.

In the present study, sensory analysis was performed by an olfactory triangular test, by comparing the three wines from the second series of fermentations. Table 5.1 shows the six sets of glasses used in the evaluation, where each wine appeared twice in the sets. Since there were two sets to compare the same wines, the responses for homologous sets were grouped, and the number of correct responses for each wine was two per taster. In this case, the total number of correct responses for each wine was 70 (35 tasters × 2 responses). According to Norm ISO 4120, (2004) the differences were considered statistically significant (p<0.05) only when the number of correct responses was higher than 31. As can be seen in Table 5.6, all of the wines showed significant differences.

Table 5.6. Number of correct answers, from a total of 70, recorded during the sensory test comparisons of the 2nd series of fermentations

Combinations	Correct answers
free cells and batch1	48
free cells and batch2	38
batch1 and batch2	43

During the sensory evaluation of wines, the panellists were also questioned as to their preference in each set of three glasses. In the six sets, each wine appeared four times, the maximum number of preferences was 140 (35 tasters × 4 possible responses). Wine produced in batch 2 recorded the greatest number of preferences, accounting for 83 votes. The wine made with free cells was the second preferred wine (70 votes), followed by the wine produced in batch 1 (60 votes). It should be noted that the preferred wine was that with the higher concentrations of the minor volatile compounds, indicating their contribution to olfactory quality.

5.4 Conclusions

Grape pomace was shown to be a suitable support for yeast immobilization and can be used for alcoholic fermentation in wine production. The duration of the fermentations were influenced mainly by the amount of the immobilization support used in each assay and also by the concentration of the SO_2 initially present in the must. However, the fermentation with the immobilized cells proved to be more rapid and efficient than the fermentation with the free cells, especially in musts with high concentrations of SO_2. Moreover it was possible to identify significant differences between the analysed wines with respect to the volatile aroma compounds.

The wines obtained with the immobilized cells showed, generally, higher concentrations of ethanol, major volatile compounds and minor volatile compounds and a higher colour intensity compared to the wines produced with the free cells. However, since the intensity of the colour decreased with the increasing number of batches, there tended to be stabilization. The sensory test suggested that the technique of cell immobilization on grape pomace applied to wine production could influence the quality of the final product. Probably, the composition of grape pomace, used as immobilization support, is one of the factors that may influence the overall process.

The sensory analysis showed noticeable olfactory differences between the wines. Those produced by immobilized cells were not compromised, since the preference of the panellists was towards the wine produced with the immobilized cells.

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6. Support selection for lactic acid bacteria immobilization

Corn cobs, grape skins and grape stems were evaluated as support materials for immobilization of the lactic acid bacteria *O. oeni*. The support materials with immobilized cells were further used in malolactic fermentation (MLF) of white wine. Viability of using the immobilized supports was evaluated in consecutive batch fermentations under different conditions of temperature, ethanol and SO_2. Additionally, the possibility of storage and operational stability of the immobilized supports was also studied. All the three supports presented large potential for immobilization of *O. oeni* cells. The consecutive batches of MLF were successfully conducted for a total period of around 5 months with the possibility of storage of the biocatalyst for 30 d in wine at 25 ºC.

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Genisheva, Z., Mussatto, S., Oliveira, J.M., Teixeira, J.A. (2013). Malolactic fermentation of wines with immobilised lactic acid bacteria – Influence of concentration, type of support material and storage conditions. *Food Chem.,* 138, 1510–1514.

6.1 Introduction

The two main fermentation processes in winemaking are alcoholic fermentation (AF) conducted by yeasts that transform sugars into ethanol and carbon dioxide, and malolactic fermentation (MLF) carried out by lactic acid bacteria that convert malic acid to lactic acid and carbon dioxide (Diviès and Cachon, 2005). MLF is a secondary fermentation that usually occurs during storage of young wines several weeks after the AF. MLF normally occurs spontaneously and is a very slow and unpredictable process that can undergo for weeks and even months, and not always give a satisfactory result (Bauer and Dicks, 2004). The wine presents unfavourable conditions for the growth of microorganisms so, even when the wine is inoculated with selected starters, there is no guarantee that the MLF will occur (Diviès *et al*., 2005; Herrero *et al*., 2004).

The implementation of MLF is very important for wines produced in cold regions as it reduces the acidity, brings biological stability and may improve the organoleptic characteristics of the product (Diviès *et al*., 2005; Kosseva *et al*., 1998). MLF determines the final quality of red and white wines and of some sparkling wines, being especially crucial for the specific organoleptic profile of Chardonnay, Burgundy white wines and Bordeaux red wines (Bauer and Dicks, 2004). In the Portuguese *Vinho Verde* wines, which are young wines, the MLF is often desirable as it partially decreases the acidity and increases the pH. A low value of pH in wines brings instability of the volatile compounds and, consequently, MLF at a suitable extent may help to preserve the aromatic characteristics of *Vinho Verde*.

In recent years, immobilized lactic acid bacteria were used for implementation of MLF in wines. According to Vila-Crespo *et al*. (2010), immobilized cell system is one of the strategies for the enhancement of malolactic fermentation in the changed climate conditions. Moreover, immobilized cell systems showed to be a good tool for the winemaking industry. Nevertheless deeper studies on this area must be done in order to ease the handling of the process and the use of this tool at the cellar (Vila-Crespo *et al*., 2010). Two main immobilization methods have been employed: encapsulation of the bacteria cells (Crapisi *et al*., 1987; Kosseva *et al*., 1998; Kosseva and Kennedy 2004; Spetolli *et al*., 1982) and attachment/adsorption onto a support (Agouridis *et al*., 2005; Maicas *et al*., 2001). The use of immobilized bacteria during MLF helps to accelerate the process and also simplifies the control of its extension. However, the material to be used as immobilization support must be carefully chosen in order to not negatively affect the final product, and should also be cheap, abundant in nature, and of food grade purity.

In this work, the lactic acid bacteria *Oenococcus oeni* was immobilized on three different natural materials (namely corn cobs, grape skins and grape stems) and used to induce malolactic fermentation in white wine. A simple, fast and effective method for immobilization of bacteria cells was used. Additionally, the viability of the biocatalyst after periods of storage in different

environments and temperatures was evaluated. The resistance of the immobilized lactic acid bacterium against the inhibitory effect of high concentration of SO_2 was also determined.

6.2 Materials and Methods

6.2.1 Inoculum preparation

A commercial strain of *Oenococcus oeni* (Uvaferm® Alpha, Lallemand) was the bacterial strain used in the experiments. The inoculum was prepared by cultivation of the bacteria in 500 mL Erlenmeyer flasks containing 200 mL of MRS medium (Cultimed). Cells were cultivated under static conditions, at 28 ºC for 48 h, being subsequently recovered by centrifugation (*RCF*=7000, 15 min), washed with distilled water and re-suspended in the fermentation medium to obtain an initial concentration of 1 g/L (dry weight).

6.2.2 Support materials for cell immobilization

Grape skin, grape stem and corn cobs were used, separately, as support materials for the bacterium immobilization and in two different concentrations 10 g/L and 30 g/L. Grape skins and grape stems, were supplied by a local winemaking industry and the corn cobs were obtained from local farmers. Before use, the support materials were washed with distilled water and dried at 60 ºC until constant weight. For further use as immobilization supports, the materials were cut and prepared according to Genisheva *et al*. (2011).

6.2.3 Cell immobilization

Fermentation runs were performed in complex culture medium with the following composition (g/L): glucose (15), yeast extract (4.0), meat extract (8.0), bacteriological peptone (10.0), $MgSO_4$ (0.2), $MnSO_4$ (0.05), sodium acetate (5.0), tween 80 (1.0), di-potassium hydrogen phosphate (2.0), di-ammonium hydrogen citrate (2.0) and malic acid (5.0). The assays were carried out in 500 mL Erlenmeyer flasks containing 200 mL of medium, bacteia cells (1g/L) and 2 g (or 6 g) of the support material. The flasks were statically incubated at 28 ºC for 10 h. Fermentations were carried out in duplicate, and samples were taken periodically for estimation of biomass, glucose and malic acid consumption, and lactic acid production.

6.2.4 Malolactic fermentations

MLF was conducted in white wine produced in laboratory conditions. Figure 6.1 is a schematic representation of the assays of malolactic fermentation carried out in the present study. Fermentation runs F6 and F7 were supplemented with sulfur dioxide in the concentration of 30 mg/L. All the assays were carried out in 500 mL Erlenmeyer flasks containing 200 mL of white wine and 7 g of each previously immobilized support. The flasks were statically incubated at 25 ºC for 17 d (except for the first and second fermentations). Fermentations were carried out

in duplicate, and samples were taken periodically for estimation of glucose, fructose and malic acid consumption, and lactic acid production. Before fermentations F5 and F7 the immobilized supports were stored as shown on Figure 6.1.

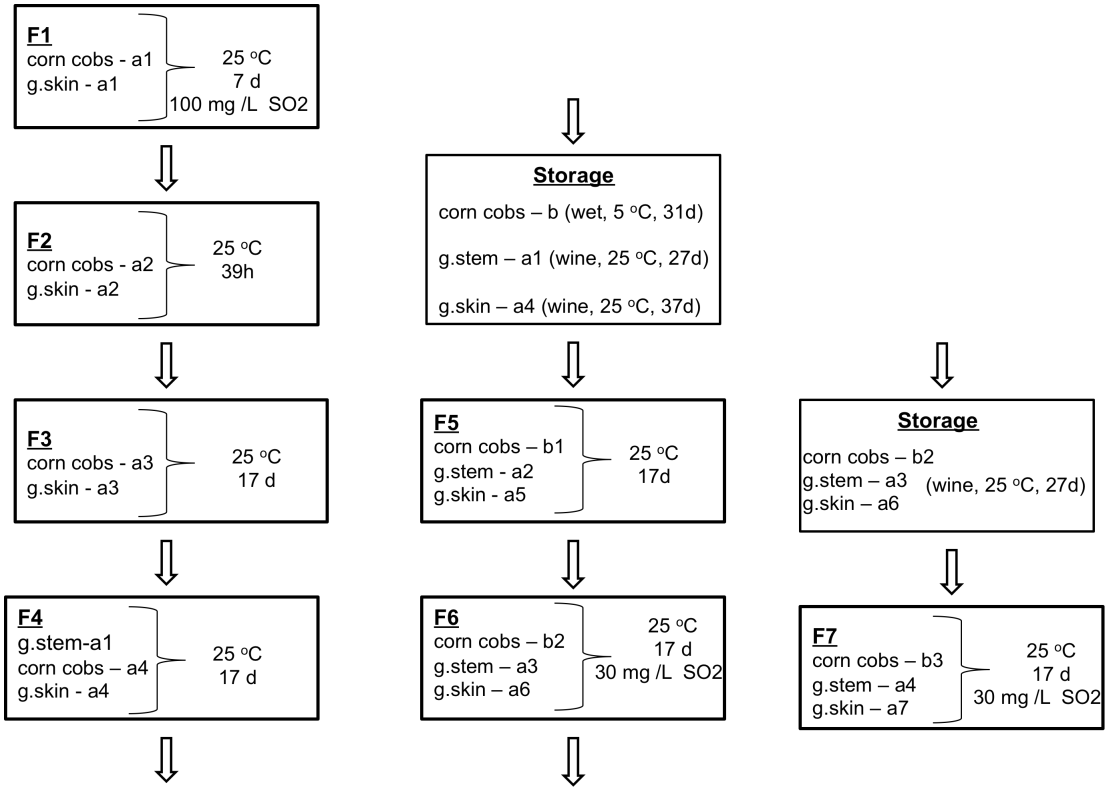

Figure 6.1. Flowchart of the malolactic fermentation assays of white wine conducted with immobilized cells.

6.2.5 Determination of immobilized biomass

The concentration of immobilized cells was determined at the end of the cell immobilization assays. Part of the immobilized material was taken aseptically from the fermentation flask and then placed in 200 mL Erlenmeyer flasks containing 20 mL of distilled water. Subsequently, the sample of biocatalyst was autoclaved for 20 min at 121 ^{o}C. The autoclaved support was separated from the liquid using a strainer and left to dry at 60 ^{o}C till constant weight. The total volatile suspended solids were calculated according to Clesceri *et al.* (1998). Corrections of the weight of volatile suspended solids for the losses of support itself were carried out by blank experiments using support without immobilized cells.

Free cells concentration in the fermentation medium was estimated by measuring the absorbance at 600 nm, which was correlated to an analytical curve (dry weight × optical density)

6.2.6 HPLC analysis

Glucose, fructose and organic acids (malic and lactic) concentrations were determined by High Performance Liquid Chromatography (HPLC) in a Jasco chromatograph equipped with a refractive index detector (Jasco 830-RI), an ultraviolet detector and a Varian Metacarb 67H column (300 mm × 6.5 mm) operated at 80 ºC. A 5 mmol/L H_2SO_4 solution was used as eluent at a constant flow rate of 0.3 mL/min.

6.2.7 Fermentation parameters

The concentration of cells immobilized on the support (X_{im}) was calculated as the ratio of cell mass immobilized on the support to the support mass. The concentration of immobilized cells in the assay (X_i) was calculated as the ratio of cell mass immobilized on the support to the volume of fermentation medium. The concentration of free cells in the assay ($X_{f.cel}$) was calculated as the ratio of cell mass to the volume of fermentation medium. Mass immobilization efficiency (Y_i) was defined as the ratio between immobilized cells and total formed cells (free + immobilized, X_t). The cell yield factor ($Y_{X/S}$) was defined as the ratio between the mass concentrations of total formed cells and the malic acid consumed. The concentration of the consumed malic acid ($C_{mal.ac}$) was calculated as the ratio of the grams of consumed malic acid per liter fermentation medium The concentration of produced lactic acid ($C_{lac.ac}$), was calculated at the 8th hour of the fermentation for immobilization. Lactic acid productivity (Q_p) was defined as the ratio between lactic acid mass concentration and the fermentation time. Malic acid conversion was calculated as the ratio between the mass concentration of the consumed malic acid and initial malic acid mass concentration.

6.2.8 Scanning electron microscopy

Micrographs of the biocatalysts (after washing with deionized water and drying for 24 h at 60 ºC) were obtained by Scanning Electron Microscopy (SEM) using a Leica Cambridge S360 microscope. To be examined, the dried samples were fixed on a specimen holder with aluminium tape and then sputtered with gold in a sputter-coater under high vacuum condition. Each sample was examined at 700-fold magnification.

6.2.9 Statistical analysis

The results were analysed by ANOVA using FAUANL software (Olivares, 1994). Tuckey´s test was used to detect significant differences between samples.

6.3 Results and discussion

6.3.1 Cell immobilization

The support materials used in the present work were chosen taking into account their nature, abundance and cost values, as well as their suitability to be used as support material for yeast cells immobilization (Genisheva *et al*., 2011). Grape skins and grape stems together with the grape seeds are known with the common name of grape pomace. The grape pomace is the biggest solid waste of the wine industry and it is of interest that an alternative use for this by-product is found. Another advantage of using grape skins and stems as immobilization supports is that they are natural products coming from the prime material, and therefore, a lesser negative effect over the final product is expected.

In the present study, the increase of the concentration of support material from 10 g/L to 30 g/L, during the immobilization assays, had a positive effect on the quantity of immobilized cells X_{im} (Table 6.1), which had the concentration doubled or even tripled. This higher affinity of the cells to the support material when the amount of support is increased is due to the biocatalyst activities of these supports. According to Genisheva *et al*. (2011) these materials provide nutrients to the medium, improving the yeast bioconversion performance.

Table 6.1. Multiple comparison analysis (Tukey's test; $p<0.05$) for the concentration of immobilized cells (X_{im}) and lactic acid ($C_{lac.ac}$), immobilization efficiency (Y_i), cell yield factor ($Y_{X/S}$), lactic acid productivity (Q_p) and total produced cell (X_t) during the malolactic fermentation by *Oenococcus oeni*

Support and concentration		X_{im} (mg/g)	Y_i %	$C_{lac.ac.}$ (g/L)	$Y_{X/S}$ (g/g)	Q_P [g/(L h)]	X_t (g/L)
Corn cobs	10 g/L	32.8 ^{b}	22.21^{cd}	32.52 ^{a}	0.71 ^{a}	4.06 ^{a}	3.75^{ab}
Grape skins		40.75 ^{b}	42.44^{bc}	27.36 ^{b}	0.61 ^{a}	3.42 ^{b}	3.43^{ab}
Grape stems		31.0 ^{b}	9.48^{d}	32.27 ^{a}	0.79 ^{a}	4.03 ^{a}	4.26^{a}
Corn cobs	30 g/L	111.0 ^{a}	68.29^{a}	14.72 ^{c}	0.81 ^{a}	1.84 ^{c}	3.43^{ab}
Grape skins		108.8 ^{a}	62.61^{ab}	14.07 ^{c}	0.69 ^{a}	1.76 ^{c}	3.16^{ab}
Grape stems		40.7 ^{b}	38.87^{bc}	14.68 ^{c}	0.59 ^{a}	1.83 ^{c}	2.42^{b}

The highest immobilization efficiency values were recorded for assays with 30 g/L of corn cobs or grape skins, with values of 68.29 % and 62.61 %, respectively. Assays with grape stems showed significant differences ($p<0.05$) in terms of immobilization efficiency (Y_i) for the two concentrations of support utilized, although there was not found statistical difference in the concentration of immobilized cells per mass of support, X_{im}. All the immobilization assays did not show significant differences ($p<0.05$) for the response of cell yield factor ($Y_{X/S}$). On the

other hand, assays using 10 g/L support material achieved higher values of produced lactic acid ($C_{lac.ac}$) and productivity (Q_p).

Immobilization of bacteria cells was additionally confirmed by SEM (Figure 6.2).

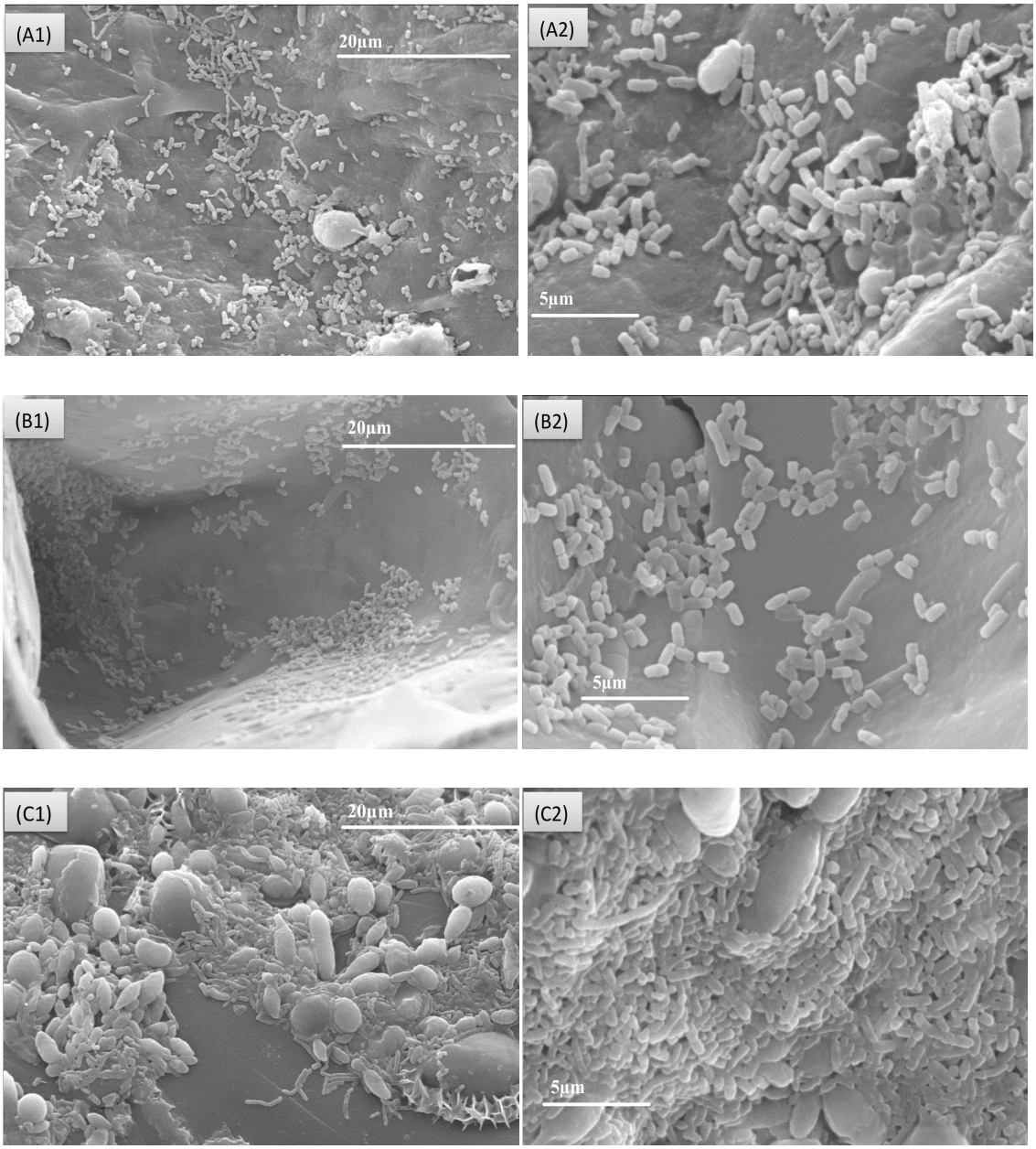

Figure 6.2. Micrographs by scanning electron microscopy (SEM) of the support materials used for cells immobilization in concentration 30 g/L. Grape stems (A1, A2) corn cobs (B1, B2) and grape skins (C1, C2) Magnification:2000-fold (A1, B1, C1) and 5000-fold (A2, B2, C2).

The SEM micrographs demonstrated that the adhesion of bacteria cells on the surface of the material was not homogenous. This fact was previously reported for the adhesion of yeast cells (Bránayik *et al*., 2004; Genisheva *et al*., 2011). Cavities (Figure 6.2, B1) and rough structures (Figure 6.2, A2) favoured the cell immobilization. It can be seen from the micrographs that for the same surface area, corn cobs (Figure 6.2, B1) and grape skins (Figure 6.2, C1), loaded much

more bacterial cells than grape stems (Figure 6.2, A1), and that is in accordance with the previous results.

Figure 6.3 shows the results obtained during MLF assays carried out with and without immobilized cells. As can be seen in this figure, fermentations with immobilized cells were twice faster than fermentations with free cells. This is in agreement with our previous study, which demonstrated also that immobilized cells improved the fermentation rates as well as the efficiency of bioconversion (Genisheva *et al*., 2011). In the presence of the support material, the production of free biomass is higher than in the fermentations containing only free cells, demonstrating that the support contributes for a better performance of the bacteria (Genisheva *et al*., 2011).

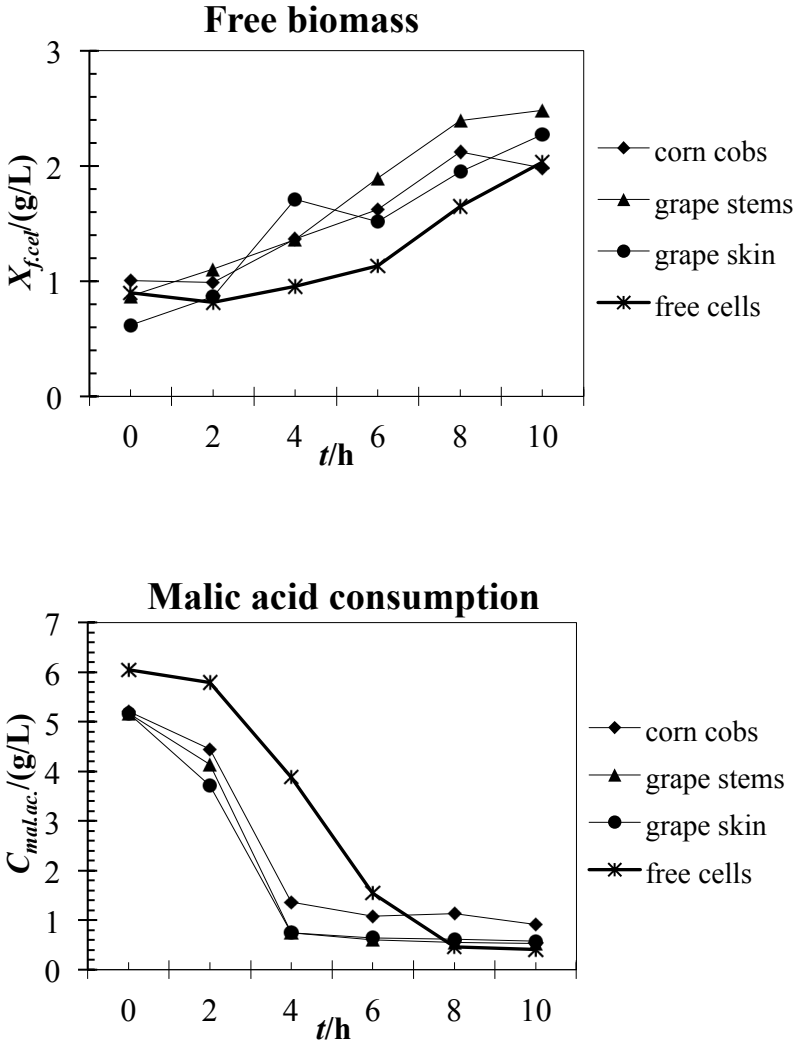

Figure 6.3. Malic acid consumption ($C_{mal.ac}$) and concentration of free biomass ($X_{f.cel}$) during the immobilization runs in presence of 30 g/L support material compared with free cell assays.

In summary corn cobs and grape skins in amounts of 30 g/L were the best support materials for *O. oeni* immobilization, since they immobilized the highest amount of cells (111.0 mg/g and

108.8 mg/g, respectively). However, fermentation with cells immobilized on 10 g/L of corn cobs and grape stems gave the highest productivity in lactic acid, 4.06 g/(L h) and 4.03 g/(L h), respectively. As a whole, fermentations with bacteria immobilized on 10 g/L support achieved more significant concentrations of lactic acid than with bacteria immobilized in 30 g/L of support. Additionally, malic acid consumption was faster in the fermentations with immobilized cells compared to fermentations with free cells (Figure 6.3).

6.3.2 Consecutive malolactic fermentations

The bacteria cells previously immobilized on 30 g/L of corn cobs, grape skins or grape stems were used for conducting malolactic fermentation in white wine. Figure 6.1 shows the MLF assays conducted with the different immobilized supports. All fermentation assays were conducted in white wine with concentrations of malic acid around 3.5 g/L. In the total, eight series of MLF were done, which were named from F1 to F7 (Figure 6.1). For the different supports, different numbers of consecutive batch fermentations were made. At the end of fermentation F4 new corn cobs-b (b-second immobilization of corn cobs) was immobilized and further used in the consecutive malolactic batch fermentations. Four consecutive batches were done with immobilized corn cobs-a (corn cobs-a1, a2, a3 and a4), and other 3 consecutive batches were made with immobilized corn cobs-b (corn cobs-b1, b2 and b3).

The bacteria activity may be affected by several parameters, being the most important the ethanol concentration and wine pH, fermentation temperature, and level of sulfur dioxide (Ribéreau-Gayon *et al*., 2006). In order to test the influence of different inhibitory factors over the performance of *O. oeni* immobilized on corn cobs and grape skins (the materials that gave better results during the immobilization), the biocatalysts were placed into a white wine with the following growth inhibitors for the bacteria cells: ethanol 9 % v/v, 20 mg/L free SO_2 and 100 mg/L total SO_2. The flasks were incubated at 25 ºC since it has been reported that the growth of *O. oeni* is inhibited and the malolactic fermentation is slower at temperatures of 25 ºC or above (Ribéreau-Gayon *et al*., 2006). Guzzo *et al*. (1998) demonstrated that in the presence of 15 mg/L of free SO_2 most of the cells of *O. oeni* died within 3 h. The growth of the bacteria is inhibited in environments richer in ethanol (above 6 % v/v), being difficult at or above 13% v/v, 14% v/v (Ribéreau-Gayon *et al*., 2006). For evaluating the viability of the immobilized cells after their prolonged exposure to these conditions, the biocatalyst from assays F1 were separated aseptically from the liquid media, washed with distilled sterilized water and placed in a new wine without sulfites at 25 ºC for 39 h (F2). Fermentation activity was noticed almost instantly. The conversion of malic acid in the MLF with immobilized corn cobs-a2 was of 5.8 %, while in assays with immobilized grape skins were of 33.0 % (Table 6.2). These results show that the immobilized cells of *O. oeni* are highly tolerant against inhibitors.

Table 6.2. Malic acid conversion (%), ± standard deviation during the consecutive MLF by immobilized *O. oeni* on different support materials

support/batch	F1	±	F2	±	F3	±	F4	±	F5	±	F6	±	F7	±
corn cobs	0	0.0	5.8	0.2	70.9	23.5	23.4	0.0	23.8	4.2	9.5	0.7	50.4	12.5
grape skin	0	0.0	33.0	7.4	49.5	4.2	86.5	0.7	62.8	3.9	84.6	0.4	38.6	23.3
grape stem	-		-		-		74.8	11.6	65.2	5.5	63.0	18.6	6.4	3.2

Once the malic acid conversions in fermentation assays from series F2 were relatively low, the fermentation time was fixed in 17 d in the subsequent batch series. During the series F3, corn cobs-a3 and grape skins-a3 were placed in a new wine for 17 d at 25 ºC. The obtained results for the malic acid conversion were of 70.9 % and 49.5 % for assays in presence of corn cobs and grape skins, respectively (Table 6.2). F4 fermentation series were conducted with cells immobilized on grape stems-a1 (batch1), grape skins-a4 (batch4) and corn cobs-a4 (batch4). The fermentations lasted 17 d and the obtained malic acid conversion was 74.8 %, 86.5 % and 23.4 %, respectively (Table 6.2). Concerning fermentations with immobilized cells an important aspect stands up, *i.e.* the storage of an immobilized support for a further use. To verify if the chosen supports are suitable for storage at different conditions and periods of time, at the end of F4 the three immobilized supports were stored at different conditions. Corn cobs were aseptically removed from the liquid and stored at 5 ºC for 31 d. Grape skins and grape stems were stored in wine from the previous MLF at 25 ºC for 37 d and 27 d, respectively (Figure 6.1). After the storage, all the supports were washed with sterilized water and placed in a new wine for a new series of fermentations (F5), which was maintained at 25 ºC for 17 d. A slight decrease in the malic acid conversion in the assays with immobilized grape skins and grape stems was observed, while the malic acid conversion was practically maintained in the assays with immobilized corn cobs. These results reveal that storage slightly affected the fermentation performance of cells immobilized on grape skins and grape stems, but not of cells immobilized on corn cobs "hidden" in the porous like surface of the corn cobs, where the biggest loads of cells are found (Genisheva *et al*., 2011).

In the subsequent series of fermentations (F6) the immobilized supports were exposed to 30 mg/L of free SO_2. Assays with grape skins were not negatively affected by the sulfur dioxide but on the contrary, there was a noticed increase of the malic acid conversion attaining a value similar to that achieved in F4 assays. The level of SO_2 used in this experiment had no effect on malic acid conversion by *O. oeni* immobilized on grape stems, while assays with *O. oeni* immobilized on corn cobs showed strong decrease of the malic acid conversion, (Table 6.2). Then, in the next stage of the study it was decided to evaluate the combined effect of storage of the immobilized supports and presence of free SO_2 (30 mg/L) in the fermentation media. At the

end of F6 series, all the supports were stored in wine at 25 ºC for 30 d. After storage, the supports were separated from the liquid, washed with distilled sterilized water and placed in a new wine with 30 mg/L free SO_2, at 25 ºC for 17 d (fermentation series F7). The obtained results for malic acid conversion were as follows: corn cobs > grape skin > grape stems (Table 6.2). It was then concluded that cells immobilized on corn cobs were more protected from the influence of the inhibitory conditions than cells immobilized in the other support materials, showing previous adaptation to the SO_2 present in the wine. Cells immobilized on grape skins and grape stems were strongly affected by the high doses of SO_2, combined with previous storage of the supports. The F7 fermentation assays were extended till 30 d of fermentations and the results showed a complete malic acid conversion (100 %) in the assays with cells immobilized on corn cobs, 75 % conversion for cells immobilized on grape skins and 82.9 % conversion for cells immobilized on grape stems (results not shown). These results suggest that the combined effect of the factors storage of the support and the presence of SO_2 in the medium did not prevent the malic acid consumption, but just slowed it down.

The results obtained in this study reveal also that the support materials used for the cells immobilization without any previous treatment, have longer operation stability when compared to delignified cellulosic material (Agouridis *et al*., 2005), being also of lower cost due to not requiring treatment prior to their use in the fermentation. In summary, *O. oeni* cells immobilized on corn cobs-b were able to conduct consecutive MLF for a total period of 150 d (3 batches), on grape stems for 174 d (4 batches) and on grape skins for 192 d (7 batches). These results are of large interest since they allow a better control and conduction of the malolactic fermentation process.

6.4 Conclusions

Corn cobs and grape skins, prepared in a culture media at the concentration (mass support per volume media) of 30 g/L, were the best support materials for *O. oeni* immobilization. Immobilized bacteria cells were more resistant against the inhibitory effect of high concentrations of ethanol, SO_2 and elevated temperatures. Cells immobilized on corn cobs were strongly affected from high concentration of free SO_2 (30 mg/L) present in the wine; however, once the cells were adapted to the presence of SO_2, there was not reduction of the malic acid conversion. Assays with cells immobilized on grape skin and grape stems were not negatively affected by the presence of 30 mg/L of SO_2 in the wine. Nevertheless, previous storage of the biocatalyst at 25 ºC for 27 d, combined with the presence of 30 mg/L of SO_2 had a strong negative effect over the malic acid conversion. Bacterial cells immobilized on corn cobs, grape skin and grape stems are capable to perform consecutive MLF for long periods of time, at least for 5 months. The immobilized supports can be stored for at least 30 d to 37 d.

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7. Continuous winemaking with immobilized cells - integrated process

An integrated winemaking process – including alcoholic and malolactic fermentations operated continuously – was developed. For the continuous alcoholic fermentation, yeast cells were immobilized either on grape stems or on grape skins, while bacteria cells used for conducting continuous malolactic fermentation were immobilized on grape skins. The produced wines were subjected to chemical analysis by HPLC (ethanol, glycerol, sugars and organic acids) and by gas chromatography (major and minor volatile compounds). The results revealed that the wine produced by the integrated process had a good quality.

The information presented in this chapter was submitted for publication.

Genisheva, Z., Mota, A., Mussatto, S., Teixeira, J.A. Oliveira, J.M. Integrated continuous winemaking process involving sequencial alcoholic and malolactic fermentations.

7.1 Introduction

The two most important processes in wine production are alcoholic fermentation (AF), conducted by yeasts, and malolactic fermentation (MLF), conducted by bacteria. During the alcoholic fermentation the sugars of grape must are transformed mainly to ethanol and carbon dioxide; additionally, a myriad of by-products are formed. Malolactic fermentation is a secondary fermentation that reduces the acidity and brings biological stability to the wines; moreover, it improves the organoleptic characteristics of the product (Ribéreau-Gayon *et al*., 2006).

In traditional winemaking the fermentation processes are conducted in discontinuous mode, *i.e*. in batch. The seasonality of the raw-material, the grapes, defines largely the organization of this sector of activity and also, the structure of the wine cellars. However continuous processes are known to be advantageous over batch processes. The continuous process is simpler to operate with low energy requirements; allowing almost complete utilization of the substrates and lowering the operating costs. Moreover, capital costs are reduced, with the possibility to obtain higher rates of production by using small bioreactors in the process. Superior productivities may be achieved by employing high concentrations of yeast or bacterial cells within the bioreactor. Nevertheless, a conventional continuous process has limitations in the maintenance of high cell concentrations in the bioreactor (Margaritis and Kilonzo, 2005). To overcome this difficulty, immobilized yeast or bacteria cell systems provide high cell density with high flow rates that results in short residence times (Verbelen *et al*., 2006). Reactors with immobilized cells have shorter fermentation times, higher productivity and operational stability of the cells, as well as easier downstream processing.

When dealing with immobilized cell systems it is of a big importance to choose the proper reactor type. This decision depends on the type of immobilization and type of support used, as well as on mass transfer requirements and conditions of the process. For continuous AF in wine production multiphase reactors are used, including packed bed reactor, fluidized bed reactor, bubble column and air-lift reactor (Kourkoutas *et al*., 2004; Verbelen *et al*., 2006). Packed bed reactor is among the most used for wine production with immobilized cells in continuous mode of operation (Kourkoutas *et al*., 2002a and 2002b). In this type of reactor the immobilized cells are packed inside the reactor and a current of fermentation media is passed upflow (flooded bed reactor) or downflow (trickle-bed reactor) (Larachi *et al*., 1997).

Most of the available data published about immobilized cell systems used in winemaking concerns batch processes, and in a less extent continuous alcoholic fermentation. Natural materials such as fruit pieces of apple, quince, pear, guava and watermelon (Kourkoutas *et al*., 2002a and 2002b; Mallios *et al*., 2004; Reddy *et al*., 2006 and 2008), whole grains of corn,

wheat and barley (Kandylis *et al*., 2010, 2012a and 2102b) or residues of the wine industry (Genisheva *et al*., 2012; Mallouchos *et al*., 2002) are reported as supports for cell immobilization and further implied in batch winemaking. Most of these immobilized cell systems were found to be with good operational stability. In continuous alcoholic fermentation, for winemaking, the used yeast cells are frequently immobilized on natural organic and inorganic materials. Immobilized cell systems using natural organic materials such as gluten pellets (Sipsas *et al*., 2009) resulted in wines with improved quality. Inorganic materials like *kissiris* and γ-alumina, are cheap, abundant and can be regenerated and reused, however this materials were considered undesirable as they leave mineral residues in the final product (Bakoyianis *et al*., 1997; Loukatos *et al*., 2000).

The immobilization methods mostly used for bacteria cell immobilization in malolactic fermentations are entrapment (Kosseva *et al*., 1998) and attachment to natural materials (Agouridis *et al*., 2008; Genisheva *et al*., 2013a) There are few available articles for continuous malolactic fermentation of wines conducted with immobilized cells (Crapisi *et al*., 1987). Moreover, according to what we know, so far no works were published about an integrated continuous process of winemaking.

The main objective of this study is the integration of both alcoholic fermentation (AF) and malolactic fermentation (MLF) in a sequential continuous winemaking process. To achieve this global goal, both AF and MLF were implemented firstly in distinct packed bed reactors operating with immobilized *Saccharomyces cerevisiae* and *Oenococcus oeni*, respectively.

7.2 Materials and Methods

7.2.1 Inoculum preparation

A commercial *Saccharomyces cerevisiae* strain (Lalvin QA23*,* Proenol*)* was used in the alcoholic fermentation experiments. The inoculum was prepared by cultivation of the yeast in 500 mL Erlenmeyer flasks containing 200 mL of YPD medium with the following composition (g/L): yeast extract (10), peptone (20) and glucose (20). Cells were cultivated under static conditions, at 30 ^{o}C for 24 h, being subsequently recovered by centrifugation (*RCF* = 7000, 20 min), washed with distilled water and resuspended in the fermentation medium to obtain an initial concentration of 1 g/L (dry weight).

A commercial strain of *Oenococcus oeni* (Uvaferm® Alpha, Lallemand) was the bacterial strain used in the of malolactic fermentation experiments. The inoculum was prepared by cultivation of the bacteria in 500 mL Erlenmeyer flasks containing 200 mL of MRS Broth medium (Cultimed, Panreac, Barcelona). Cells were cultivated under static conditions, at 28 ^{o}C for 48 h, being subsequently recovered by centrifugation (*RCF* = 7000, 10 min), washed with

distilled water and resuspended in the fermentation medium to obtain an initial concentration of 1 g/L (dry weight).

7.2.2 Support materials for cell immobilization

Grape skins and grape stems, separately, were used as support materials for cell immobilization. These supports were supplied by a local winemaking company, being washed with distilled water and dried at 60 ºC until constant weight. Then, supports were sterilized for 20 min at 121 ^{o}C, before use.

7.2.3 Media composition for fermentation assays

Synthetic culture medium used in the alcoholic fermentation assays was composed by (g/L): glucose (120), yeast extract (4), $(NH_4)_2SO_4$ (1), KH_2PO_4 (1), $MgSO_4$ (5). Synthetic culture medium used in the malolactic fermentation had the following composition (g/L): glucose (15), yeast extract (4.0), meat extract (8.0), bacteriological peptone (10.0), $MgSO_4$ (0.2), $MnSO_4$ (0.05), sodium acetate (5.0), tween 80 (1.0), di-potassium hydrogen phosphate (2.0), di-ammonium hydrogen citrate (2.0) and malic acid (4.0).

The grape must used for alcoholic fermentations was obtained from a mixture of white grape varieties from the Appellation of Origin *Vinhos Verdes* region. The used wines for malolactic fermentation were produced in laboratory conditions and had an initial concentration of malic acid around 4 g/L. The grape must and wine were kept at 4 ºC, before use. The choice of synthetic medium in the initial fermentations avoided difficulties with supply and storage of grape must.

7.2.4 Reactors preparation

Continuous alcoholic and malolactic fermentation assays were performed in distinct cylindrical tower packed bed reactors (7.2 cm inside diameter) with a total volume of about 1750 mL. Two sampling ports were available at 20 cm and 37 cm height, corresponding to volumes of the empty bed of 814 mL and 1506 mL, respectively (Figure 7.1). Both reactors were operated in upward flow mode.

Before use, the reactors were sterilized with sodium hypochlorite solution (1.5 % active chlorine) during at least 4 d prior to fermentations (Bránayik *et al*., 2006). Then, the reactors were washed five times with sterilized water before filling with the sterilized support. At the bottom of each reactor, 1 cm height of glass beads (6 mm diameter) was placed to allow a regular repartition of the feeding medium in the whole section of the tower. Then, the reactor for continuous alcoholic fermentation was packed with grape stems (60 g and 90 g) or with grape skins (125 g and 260 g) in sterile conditions in the flow chamber. Similarly, packed bed reactor

for continuous malolactic fermentation was filled in with 260 g of grape skins. The assays with 60 g, 90 g and 125 g of material were carried out using the first sampling port, *i.e.* at 20 cm height; the assays with 260 g of support were performed using the total available volume at 37 cm height. The supports were restricted with an iron nets placed above the glass beads and above the support itself.

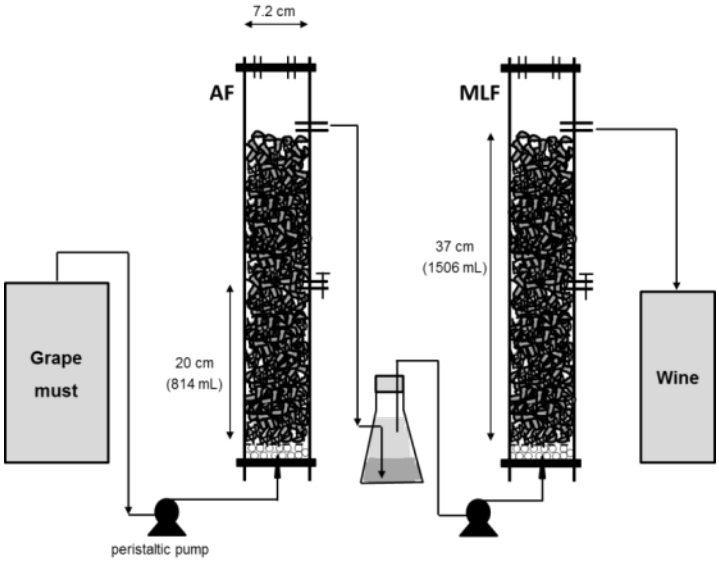

Figure 7.1. Schematic representation of the integrated process of continuous winemaking.

7.2.5 Fermentation assays

A schematic representation of the assays carried out in the present study is depicted in Figure 7.2. Initially, for cells immobilization, the reactors were operated in batch mode. The reactors were charged with 1 L of synthetic medium and about 50 mL of yeast or bacteria cell suspension prepared as described in the inoculum preparation subsection. Then, after 48 h of immobilization in batch process, the reactors were switched to a continuous mode, and operated at different dilution rates (Figure 7.2). After all the operational conditions of the reactors were established and the processes were stabilized, the synthetic medium was replaced by grape must for alcoholic fermentation with total sugar content ≈ 200 g/L, and wine for malolactic fermentation. Continuous AF and MLF were conducted initially in separate, after optimization of the processes conditions the two reactors were linked together.

Aiming at searching the optimum conditions for the production of wine, continuous alcoholic fermentation was conducted in a packed bed reactor at different operational conditions. Two different support materials, grape stems and grape skins were evaluated for immobilization of yeast cells. Moreover, these supports were used in different amounts (Figure 7.2). Grape stems (in amounts of 60 g and 90 g) and grape skins at the amount of 125 g were used in packed bed reactor with empty bed volume of 814 mL. With the increasing of the amount of the grape skins from 125 g to 260 g the second sampling port was used, corresponding to an empty bed volume

of the reactor of 1506 mL. Continuous malolactic fermentations were conducted in a second packed bed reactor (with equal dimensions) with a volume of the liquid of 1506 mL and 260 g of grape skins, at different dilution rates.

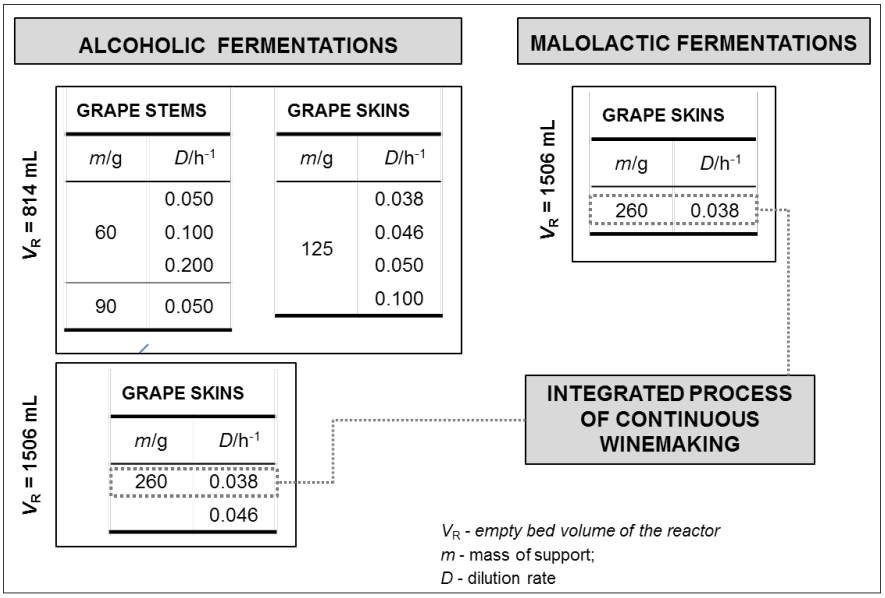

Figure 7.2. Flowchart of the continuous fermentation assays conducted with immobilized cells.

7.2.6 Free and immobilized cells determination

Immobilized yeast cells concentration was determined by counting the yeast cells on a Neubauer chamber at the fermentations' end after washing the biocatalyst by agitation (120 min^{-1}) with 30 g/L NaOH solution, for 24 h at 30 ºC , according to Genisheva *et al.* (2011).

Death/live cells were determined after detachment of the cells by vigorous agitation of 0.5 g of the support with 30 g/L solution of NaCl, for 30 min. Then, the liberated cells were further stained with methylene blue and the dead/live cells were counted on a Neubauer chamber.

The concentration of immobilized bacterial cells was determined at the end of fermentation assays. About 3.5 g of material (wet weight) were placed in a 200 mL Erlenmeyer flask containing 20 mL of distilled water. Subsequently, it was autoclaved for 20 min at 121 ^{o}C. The autoclaved support was separated from the liquid using a strainer and left to dry at 60 ^{o}C till constant weight. The total volatile suspended solids, remained in water, were determined by gravimetry according to Clesceri *et al.* (1998). A blank experiment using support without immobilized cells was used to correct eventual losses of material during the autoclaving procedure.

Free yeast and bacterial cells concentrations, present in the fermentation medium, were estimated by measuring the absorbance at 600 nm, which was correlated to a calibration curve (dry weight × absorbance).

7.2.7 Fermentation parameters

The concentration of immobilized cells (X_{im}) was calculated as the ratio of the dry weight of cells to the dry weight mass of the support. The mass concentrations of the consumed malic acid ($C_{mal.ac}$), the produced lactic acid ($C_{lac.ac}$) and the produced acetic acid ($C_{ac.ac}$) were calculated respectively as the ratio of the mass of consumed malic acid or the produced lactic acid and acetic acid, per litre of fermentation medium. The conversion of malic acid ($\eta_{mal.ac}$) was determined as the ratio between the consumed malic acid and the initial malic acid. The mass concentrations of glucose (C_{gl}) and fructose (C_{fr}) were calculated as the mass of glucose and fructose per litre of fermentation medium. The alcohol strength (C_{et}) was calculated as the volume of ethanol present in 100 volumes of the fermentation product. The dilution rate (D) was defined as the ratio between the volumetric flow rate and the liquid phase volume of the packed reactor.

7.2.8 General physicochemical analysis

Total acidy (TA) and free SO_2 concentrations were measured by titration according to the Methods OIV-MA-AS313-01 and OIV-MA-AS323-04A, respectively (OIV, 2012).

7.2.9 HPLC analysis

Glucose, fructose, ethanol, glycerol and organic acids (citric, tartaric, malic, succinic lactic and acetic) concentrations were determined by high performance liquid chromatography (HPLC) in a Jasco chromatograph equipped with a refractive index detector (Jasco 830-RI), an ultraviolet detector and a Varian Metacarb 67H column (300 mm × 6.5 mm) operated at 80 ºC. A 5 mmol/L H_2SO_4 solution was used as eluent at a constant flow rate of 0.3 mL/min. These analyses were made in duplicate.

7.2.10 Gas-Chromatographic analysis

Major volatile compounds were directly analysed after adding 410 μg of 4-nonanol (internal standard – IS) to 5 mL of wine. A Chrompack CP-9000 gas chromatograph equipped with a split/splitless injector, a flame ionization detector (FID) and a capillary column, coated with CP-Wax 57CB (50 m × 0.25 mm; 0.2 μm film thickness, Chrompack), was used. The temperatures of the injector and the detector were both set to 250 °C. The oven temperature was initially held at 60 °C, for 5 min, then programmed to rise from 60 °C to 220 °C, at 3 °C/min, and finally maintained at 220 °C for 10 min. The carrier gas was helium 4× (Praxair) at an initial flow rate of 1 mL/min (125 kPa at the head of the column). The analyses were performed by injecting 1 μL of sample in the split mode (15 mL/min). The quantification of major volatile compounds, after the determination of the detector response factor for each analyte, was performed with the

software Star-Chromatography Workstation version 6.41 (Varian) by comparing test compound retention times with those of pure standard compounds.

Minor volatile compounds were analyzed by GC-MS after extraction of 8 mL of wine with 400 μL of dichloromethane, spiked with 3.28 μg of 4-nonanol (IS), according to the methodology proposed by Oliveira *et al.*(2006). A gas chromatograph Varian 3800 with a 1079 injector and an ion-trap mass spectrometer Varian Saturn 2000, was used. A 1 μL injection was made in splitless mode (30 s) in a Varian Factor Four VF-Wax ms (30 m × 0.15 mm; 0.15 μm film thickness) column. The carrier gas was helium 4× (Praxair) at a constant flow rate of 1.3 mL/min. The detector was set to electronic impact mode with an ionization energy of 70 eV, a mass acquisition range from 35 *m*/*z* to 260 *m*/*z* and an acquisition interval of 610 ms. The oven temperature was initially 60 ^{o}C for 2 min and then raised from 60 ^{o}C to 234 ^{o}C at a rate of 3 ^{o}C/min, raised from 234 ^{o}C to 250 ^{o}C at 10 ^{o}C/min and finally maintained at 250 ^{o}C for 10 min. The temperature of the injector was maintained at 250 ^{o}C during the analysis time and the split flow was maintained at 30 mL/min. The identification of compounds was performed using the software MS WorkStation version 6.9 (Varian) by comparing their mass spectra and retention indices with those of pure standard compounds. The minor compounds were quantified in terms of 4-nonanol equivalents only. All the analyses of volatiles were carried out in triplicate.

7.2.11 Statistical analysis

The results were analysed by ANOVA, using FAUANL software (Olivares, 1994). Fisher's Least Significance Difference (LSD) multiple comparison test was used to detect significant differences between samples.

7.3 Results and discussion

In previous studies, Genisheva *et al*. (2012 and 2013a) showed the possibility of conducting, in batch mode of operation, alcoholic and malolactic fermentations with *S. cerevisiae* and *O. oeni* cells immobilized either on grape stems or on grape skins. The present study evaluates the possibility of conducting the same fermentations but in a continuous mode of operation. In the first part of this work, studies with immobilized yeast cells to carry out the AF in wine production were made. Different amounts of two different supports (grape stems and grape skins) were used, operating at different dilution rates. In the second part assays with immobilized bacteria cells for malolactic fermentation, were conducted. Finally, the integration of the two continuous fermentative processes, in a whole winemaking procedure, was studied. Initially, the studies were conducted with synthetic medium and later grape must and wine were

used for alcoholic and malolactic fermentations, respectively. All analyses were made in duplicate after the stationary state of the continuous process was attained.

7.3.1 Continuous alcoholic fermentations

Continuous alcoholic fermentation assays were made with different concentrations of immobilized support, as well as with different dilution rates. The obtained results are presented in Table 7.1.

Table 7.1. General characteristics of the alcoholic fermentation assays with immobilized *S. cerevisiae* and multiple comparison analysis (Fisher's test; $p<0.05$), including standard deviation (*sd*)

support	$\frac{m}{g}$	media	$\frac{t}{d}$	$\frac{D}{h^{-1}}$	$\frac{C_{gl.in}}{g/L}$	$\frac{C_{gl.fin}}{g/L}$	$\frac{C_{et}}{\%\ vol.}$	*sd*	$\frac{X_{im.T}}{mg/g}$	*sd*	$\frac{X_{im.B}}{mg/g}$	*sd*
Grape stems	60	S	5	0.050	120	42	6.0^{fg}	0.3				
		S	2	0.100	120	92	2.1^{h}	0.1				
		S	1	0.200	120	110	1.0^{h}	0.0	5.1	2.8	7.7	3.1
	90	S	25	0.050	200	130	4.3^{g}	0.0	8.3	2.4	19.4	0.5
Grape skins	125	S	7	0.038	200	24	11.7^{ab}	0.3				
		S	7	0.046	200	21	11.4^{bc}	0.9				
		S	42	0.050	200	29	9.9^{cd}	0.2				
		S	43	0.058	200	36	11.1^{bc}	0.8				
		S	6	0.100	200	90	8.6^{de}	0.6	205.8	12.2	537.2	54.6
	260	S	3	0.038	120	0	7.1^{ef}	0.2				
		S	5	0.046	120	3	8.6^{de}	0.6				
		S	5	0.038	200	2	12.7^{a}	0.3				
		S	19	0.046	200	42	11.2^{bc}	0.0				
		M	47	0.038	200	2	13.1^{a}	0.1	1476.8	128.1	2256.9	18.9

a, b, c, d, e, f, g, h – for each column, values with the same letters mean no significant difference at 95 % confidence level; S– synthetic media; M– grape must; *m*– mass of the support; *t*– total fermentation time of the assay; $C_{gl.in}$– initial glucose concentration; $C_{gl.fin}$– final glucose concentration; $X_{im.T}$– concentration of immobilized cells at the top of the reactor; $X_{im.B}$– concentration of immobilized cells at the bottom of the reactor.

The first two continuous fermentations were carried out with 60 g and 90 g of grape stems. The other two fermentation assays were carried out with grape skins, respectively 125 g and 260 g. In all assays with grape skins these were hold down with small amount of grape stems, as well as with an iron net. Firstly the packed bed reactor worked in batch mode, with synthetic medium, for better cell immobilization. After 48 h the reactor was switched to a continuous mode of operation.

The assays with 60 g of immobilized grape stems were conducted with three different dilution rates (0.05 h^{-1}, 0.10 h^{-1} and 0.20 h^{-1}), the most effective conditions being obtained at D = 0.05 h^{-1}. At this dilution rate, the ethanol production (6 % vol.) showed the best values

compared to the two other dilution rates used. The residual glucose concentration was rather high, 42 g/L, and the amount of immobilized cells was low. According to this, the next assay was carried out with 90 g of grape stems at D value of 0.05 h^{-1}. In this assay the initial sugar concentration was increased to 200 g/L, which theoretically would give an alcoholic strength to the final product of about 13 % vol. However, the obtained results were much lower (4.3 % vol.) The production of ethanol (5 % vol.) was low, which is in agreement with the high final glucose concentration observed (130 g/L).

To overcome this problem, grape skins were used as support material in a new series of continuous AF assays. In the first assay the total mass of the support was set to 125 g to obtain higher concentrations of immobilized cells. The initial concentration of glucose was 200 g/L and 5 different dilution rates were studied (Table 7.1). The best result for ethanol production (11.7 % vol.) was registered for D = 0.038 h^{-1}. Good results were obtained also when the reactor was operated at D = 0.046 h^{-1}. Nevertheless, the minimum final concentration of glucose, 21 g/L, was still high (Table 7.1). The obtained quantities of immobilized cells (205.8 mg/g on the top of the packed bed and 537.2 mg/g at the bottom of the packed bed) were much higher than in the previous assay with 90 g of grape stems as support (8.3 mg/g and 19.4 mg/g, on top and bottom of the packed bed, respectively.

In attempt to reach higher immobilized cell load in the reactor and to decrease the final glucose concentration, the last continuous alcoholic fermentation was carried out with 260 g of immobilized grape skins, using the total bed reactor liquid volume, *i.e.* 1506 mL. Firstly, at initial sugar concentration of 120 g/L, the obtained results for ethanol production were low, 7.1 % vol. for D = 0.038 h^{-1} and 8.6 % vol. for D = 0.046 h^{-1}. Initially, the two first assays with synthetic medium were conducted with decreased concentration of glucose, 120 g/L, as it was observed previously (data not shown) that cell adhesion to the support, *i.e.* immobilization, is faster. After this initial start-up for a better immobilization of yeast cells, the initial glucose concentration was increased to 200 g/L. In result, the ethanol production increased as follows: 11.2 % vol. for D = 0.046 h^{-1} and 12.7 % vol. for D = 0.038 h^{-1}.

As the reactor operation remain stable for more than 1 month (since the beginning of the assay with 260 g of grape skins) the synthetic medium was changed to grape must. At these conditions and after achieved the steady state, the obtained results showed improved ethanol production of 13.1 % vol. The reactor operated with grape must for 47 d. At a dilution rate of 0.038 h^{-1}, the final glucose concentration reached low values of 2 g/L, *i.e.* a dry white wine was produced. Moreover, the concentration of immobilized cells calculated at the end of the fermentation demonstrated high cell load in the entire reactor (1476.8 mg/g on the top and 2256.9 mg/g on the bottom).

During the continuous alcoholic fermentation with immobilized cells in the packed bed reactor, working at D = 0.038 h^{-1}, the flow rate of wine production was ≈ 960 mL/d. being the AF completed in 26.3 h. In a previous study, Genisheva *et al.* (2013b) using immobilized yeast cells on grape skins for wine production, in batch mode, at least 4 d were needed to complete the alcoholic fermentation in a 1 L containers. Comparing these results it is clear that continuous alcoholic fermentations with immobilized yeast cells are about 4 times more productive than fermentations in the traditional batch mode.

7.3.2 Continuous malolactic fermentations

On the bases of the previous study of continuous alcoholic fermentation, for conducting continuous malolactic fermentations 260 g of immobilized support with bacteria cells were used, operating at a dilution rate of 0.038 h^{-1}. Firstly, *Oenococcus oeni* bacterial cells were allowed to immobilize in the previously sterilized grape skins, using synthetic medium. For screening the bacteria development and growth samples were taken at different time periods. After 48 h the reactor was switched to the continuous mode of operation. Two continuous malolactic fermentations were carried out. For each fermentation assay, firstly the system was supplied with synthetic medium and then it was replaced by dry white wine (Table 7.2).

In the first fermentation, the malic acid conversion in continuous fermentation with synthetic medium was 91 % (Table 7.2). However, when the wine was supplied a gradual decrease of the malolactic conversion was observed, possibly due to the low pH (2.9). At the 5th day the malic acid conversion was 21 % and after 17 d the malolactic fermentation stopped completely. These results are in agreement with those referred by Ribéreau-Gayon *et al*. (2006), which stated a pH of 2.9 as the limit for the growth of lactic acid bacteria.

Table 7.2. General characteristics of the wines obtained after continuous malolactic fermentation and multiple comparison analysis (Fisher's test; p<0.05), including standard deviation (*sd*)

Fermentation	medium	$\eta_{mal.ac.}$ %	*sd*	$C_{mal.ac}$ g/L	*sd*	$C_{lac.ac}$ g/L	*sd*
1	synthetic	91^{a}	2.6	3.6^{a}	0.1	8.6^{a}	0.0
	wine (pH 2.9)	21^{b}	1.7	0.8^{c}	0.1	8.4^{a}	0.3
2	synthetic	93^{a}	1.7	3.3^{a}	0.1	5.8^{b}	0.6
	wine (pH 3.1)	85^{a}	0.0	2.1^{b}	0.0	5.7^{b}	0.0

a, b, c – for each column, values with the same letters mean no significant difference at 95 % confidence level

In the second continuous malolactic fermentation assay, the conversion of malic acid, using synthetic medium reached 93 %. The higher pH value of the wine (3.1) in this assay facilitates the malolactic conversion (85 %). According to Ribéreau-Gayon *et al*. (2006), at pH 3.2 the bacterial growth is still very limited and malolactic fermentation only became possible at a pH

of 3.3 or higher. However immobilized bacteria cells are more resistant against inhibitors (Genisheva *et al*., 2013a and 2013b) and the pH 3.1 was high enough for the immobilized bacteria cells to conduct successfully malolactic fermentation. In those conditions the used system gave high degradation of malic acid and long term (around 1 month) operation stability.

7.3.3 Integrated continuous winemaking

Two assays of winemaking, integrating the two continuous processes were made (Figure 7.1). The packed bed reactor with immobilized yeast cells was linked to the packed bed reactor with immobilized bacteria cells, using a *kitasato* flask as a clarifier between them. The outflow of the first reactor, after sedimentation of yeast cells, was indeed the inflow of the second reactor (Figure 7.1). The whole system was operating in continuous mode. The first assay was not successful as the wine produced in the first reactor had very low pH value of 2.9 (Table 7.3). However, in the second assay the wine produced by AF had higher pH (3.1) resulting in a final wine with expected good characteristics

Table 7.3 presents some general characteristics of the wine obtained in this integrated process. In the first integrated assay, the degradation of malic acid was very poor, 0.7 %. As mentioned before, the low pH value of the wine produced in the reactor with immobilized yeast cells may justify the results; additionally, the synergic effect of the high alcoholic strength may have influence. Here, the residual sugars, glucose and fructose, present after the alcoholic fermentation were further reduced during the MLF by 15 % and 50 %, respectively. No changes in ethanol concentration were found, as expected (Agouridis *et al*., 2005).

In the second assay of the integrated process the pH of the wine produced in the first packed reactor had a pH 3.1. As a result, the degradation of malic acid in the second reactor was much higher 66.6 %. In the total, 1.6 g of malic acid were consumed by the immobilized bacteria cells, and 2.9 g of lactic acid were produced. The system was able to metabolize the malic acid, being the results comparable with previous studies (Agouridis *et al*., 2008). The concentration of sugars, glucose and fructose, also decreased and were converted partially to lactic acid. According to Genisheva *et al*. (2013a) bacterial cells immobilized on grape skins were able to diminish the concentration of malic acid from 50 % to 87 % in 17 d (in batch mode of operation). In the present study, after the system reached the steady state, the continuous malolactic fermentation using immobilized bacterial cells converted about 67 % of malic acid at a flow rate of 980 mL/d and a fermentation time of 26.3 h (*i.e.* $D = 0.038\ h^{-1}$).

Table 7.3 General characteristics of the wine obtained after alcoholic fermentation (AF) and after malolactic fermentation (MLF) including standard deviation (*sd*)

	1st assay (pH 2.9)				2nd assay (pH 3.1)			
	AF	*sd*	MLF	*sd*	AF	*sd*	MLF	*sd*
$C_{mal.ac}$/(g/L)	2.6	0.1	2.6	0.1	2.4	0.12	0.8	0.0
$\eta_{mal.ac.}$/%			0.7	0.4			66.6	1.0
$C_{ac.ac}$/(g/L)	0.6	0.0	0.6	0.0	0.4	0.04	0.6	0.0
$C_{lac.ac}$/(g/L)	1.6	0.0	1.4	0.0	1.6	0.18	4.5	0.0
C_{gl}/(g/L)	0.4	0.0	0.3	0.0	0.3	0.01	0.0	0.0
C_{fr}/(g/L)	0.9	0.0	0.4	0.0	0.8	0.04	0.0	0.0
C_{et}/(% vol.)	12.7	0.0	12.9	0.2	12.2	0.33	11.5	0.0
pH	2.9		nd		3.1		3.3	
TA/(g/L)	nd		nd		2.4		0.2	

nd– not determined; *TA* – total acidity, expressed as tartaric acid

The low concentrations of residual sugars, present after AF, were completely exhausted during MLF, while the ethanol percentage did not change after the continuous malolactic fermentation. Acetic acid is the most important volatile acid produced during MLF. A concentration of acetic acid of 0.2 g/L to 0.6 g/L contributes to the complexity of the wine aroma (Bartowsky and Henschke, 1995; Lerm *et al*., 2010). In the present study, the concentration of acetic acid rises from 0.4 g/L to 0.6 g/L (Table 7.3). It is well known that the concentration of acetic acid normally increased 0.1 g/L to 0.2 g/L after MLF (Lerm *et al*., 2010), which corroborates the obtained results. The limit for volatile acidity in white wines, which comprises essentially acetic acid, is 1.2 g/L (OIV 2012).

To fully compare the wine produced in continuous alcoholic fermentation with the wine obtained after continuous malolactic fermentation, in the integrated system, a complete characterization of the products regarding aroma volatile compounds was made. The obtained results are presented at Table 7.4 and Table 7.5. In total, 8 major volatile compounds and 19 minor volatile compounds were identified and quantified by GC-FID and by GC-MS, respectively.

7.3.4 Major volatile compounds

From the 8 major volatile compounds analysed only acetaldehyde and four higher alcohols demonstrated significant difference before and after malolactic fermentation (MLF). Moreover four compounds were found in concentrations above their perception thresholds for both samples, after AF and after MLF. Acetaldehyde increased significantly ($p< 0.05$) after MLF, which is in agreement with other published data (Agouridis *et al*. 2005 and 2008). However, in our study acetaldehyde was found in much lower concentrations compared to other studies with immobilized bacterial cells conducting MLF in batch mode (Agouridis *et al*., 2008).

Nevertheless acetaldehyde was always found above its orthonasal perception threshold of 10 mg/L (Moreno *et al*., 2005). Also, ethyl acetate was found, for all samples, in concentrations above its perception threshold of 12.3 mg/L (Escudero *et al*., 2004) and in concentrations similar to other published results (Sipsas *et al*., 2009). Ethyl acetate is considered an important contributor to the wine aroma. At low concentrations (≤100 mg/L) this compound gives desirable "fruity" aromas to the wine, however at higher concentrations it can impart a "solvent" or "nail varnish-like" aromas (Sumby *et al*., 2010). Methanol is produced from the pectins of the skin of the grapes which undergo an enzymatic conversion (Ribéreau-Gayon *et al*., 2006). Nevertheless the found concentrations for methanol in wines produced with cells immobilized on grape skins were low (24.6 g/L after AF and 23.9 g/L after MLF). Sipsas *et al.* (2009), using a packed bed reactor in continuous mode, reported methanol concentrations two times higher than those found in the present study. Also Tsakiris *et al.* (2004), using immobilized yeasts on raisins to carry out AF (in batch mode), found methanol concentrations (93.2 g/L and 86.5 g/L) 4 times higher compared to the obtained results in the present study (Table 7.4).

Table 7.4. Mean concentrations (*C*), confidence limits (p = 0.05) and aroma perception thresholds (*PT*) of the major volatile compounds at the end of alcoholic fermentation (AF) and at the end of the malolactic fermentation (MLF)

Compound	**AF**		**MLF**		*PT*/(mg/L)
	C/(mg/L)	±	*C*/(mg/L)	±	
acetaldehyde	20.9[b]	5.7	30.7[a]	7.4	10[A]
ethyl acetate	57.4[a]	16.9	66.0[a]	21.8	12.3[B]
methanol	24.6[a]	2.5	23.9[a]	10.8	668[A]
1-propanol	85.3[a]	13.9	61.5[b]	16.6	830[A]
2-methyl-1-propanol	37.4[a]	7.6	30.0[b]	7.8	40[A]
2-methyl-1-butanol	14.9[a]	3.0	11.6[b]	3.1	
3-methyl-1-butanol	102.9[a]	20.7	80.9[b]	24.2	30[A]
2-phenylethanol	6.6[a]	1.1	5.7[a]	3.3	14[C]
Total high alcohols	**245.3**	31.8	**189.7**	39.9	

a, b, c, d – for each compound, values with the same letters mean no significant difference at 95 % confidence level; **A**- Moreno *et al*., 2005; **B**- Escudero *et al*., 2004; **C**- Ferreira *et al*., 2000

Alcohols having more than two carbons and only one alcohol function are called higher alcohols. As higher alcohols are produced during AF, they are absent in grape must, but are found in wines in relatively high concentrations, reaching values above 100 mg/L (Vilanova and Oliveira, 2012). The present study shows that higher alcohols (except 2-phenylethanol) concentrations diminished significantly (p<0.05) after MLF. Similar results were published by Agouridis *et al*. (2005), using immobilized *Lactobacillus casei* cells on a delignified cellulosic material. The sum of the higher alcohols attains 245.3 mg/L after AF and 189.7 mg/L after MLF. These results are in accordance with the previous statement (Vilanova and Oliveira,

2012). Excessive concentrations of higher alcohols may give "strong" and "pungent" notes to wines (Nykänen, 1986), while levels below 300 mg/L to 400 mg/L may impart fruity character (Rapp and Versini, 1995; Swiegers *et al*., 2005).

In wines, 1-propanol is normally found in concentrations between 1 mg/L and 50 mg/L. However, in the present study 1-propanol was always found in concentrations higher than 50 mg/L, but it never reached its perception threshold of 830 mg/L (Moreno *et al*., 2005). After AF, the wine had much higher concentrations of 1-propanol and was found statistically different ($p<0.05$) from wine after MLF. Additionally, it was found in much higher concentrations than those reported by Sipsas *et al.* (2009), 23 mg/L, in wines produced continuously in a packed bed reactor with cells immobilized on gluten pellets.

The formation of higher alcohols is connected to the amino acids catabolism (Ehrlich pathway) and to the sugar metabolism of yeasts (Vilanova and Oliveira, 2012). The higher alcohol 3-methyl-1-butanol was present, in both samples, in concentrations over its perception threshold of 30 mg/L (Moreno *et al*., 2005). The sum of 2-methyl-1-butanol and 3-methyl-1-butanol (117.8 mg/L) before MLF was higher compared to other published results of 75 mg/L for wine produced in continuous packed bed reactor (Sipsas *et al*., 2009). According to Vilanova and Oliveira (2012), 2-methyl-1-butanol and 3-methyl-1-butanol together with 2-phenylethanol are the higher alcohols that most contribute to the aroma of wine.

In general, factors that increase the fermentation rate, such as higher concentrations of yeast biomass, also increase the formation of higher alcohols. Moreover the content of higher alcohols of wine varies according to the fermentation conditions, especially the species of yeast used in the fermentation process (Ribéreau-Gayon *et al*., 2006).

7.3.5 Minor volatile compounds

From the 19 minor volatile compounds identified and quantified, 9 were found to be statistically different ($p<0.05$) in wines before and after MLF.

Fatty acids ethyl esters are formed enzymatically in a reaction between ethanol and fatty acids (Vilanova and Oliveira, 2012). Esters have similar olfactory notes bringing fruity and pleasant characteristics to the overall aroma of wines (Vilanova and Oliveira, 2012). However, changes in ester concentrations during MLF are strain specific (Sumby *et al*., 2010). All fatty acids ethyl esters, except ethyl decanoate, were found in the wine samples in concentration above their perception thresholds after AF (Table 7.5). Moreover ethyl octanoate and ethyl decanoate increased their concentration after MLF, fact that is in agreement with Lerm *et al*. (2010). Fatty acid ethyl esters are a group of compounds that had higher total concentrations in the present

wine samples. This makes sense, as the synthesis of esters is dependent on the need of yeasts to form fatty acids (Vilanova and Oliveira, 2012).

Table 7.5. Mean concentrations (*C*), confidence limits (p = 0.05) and aroma perception threshold (*PT*) of the minor volatile compounds at the end of alcoholic fermentation (AF) and malolactic fermentation (MLF)

Compound	AF		MLF		*PT*/(μg/L)
	C/(μg/L)	±	*C*/(μg/L)	±	
Fatty acid ethyl esters					
ethyl butyrate	186.5[a]	65.9	148.7[a]	13.9	20[B]
ethyl hexanoate	758.6[a]	78.6	545.3[a]	35.5	14[A]
ethyl octanoate	257.7[b]	13.7	551.9[a]	47.9	5[A]
ethyl decanoate	142.4[b]	43.9	222.0[a]	41.1	200[A]
total	**1345.2**	112.4	**1467.9**	73.7	
Ethyl esters of organic acids					
ethyl lactate	76.0[a]	22.1	32.3[b]	7.8	100 000[C]
diethyl succinate	nd		nd		100 000[C]
total	**76.0**	22.1	**32.3**	7.8	
Acetates of higher alcohols					
3-methylbutyl acetate	2050.6[a]	309.1	1371.9[b]	161.6	30[C]
hexyl acetate	57.2[a]	15.1	37.1[b]	10.8	1000[D]
2-phenylethyl acetate	170.4[a]	19.8	114.4[b]	3.1	250[C]
total	**2278.2**	310.1	**1523.4**	162.0	
Volatile phenols					
4-vinylguaiacol	4.4[a]	2.9	1.7[b]	0.6	130[E]
4-vinylphenol	nd		nd		180[E]
total	**4.4**	2.9	**1.7**	0.6	
Volatile fatty acids					
butanoic acid	36.5[a]	12.6	33.2[a]	5.2	173[A]
hexanoic acid	688.4[a]	170.0	603.4[a]	45.0	420[A]
octanoic acid	2617.0[a]	497.5	2382.3[a]	185.2	500[A]
decanoic acid	420.9[a]	296.8	150.0[b]	54.4	1000[A]
dodecanoic acid	64.9[a]	10.5	6.8[b]	2.4	10 000[C]
2-methylpropanoic acid	26.7[a]	7.5	22.1[a]	1.9	2300[A]
2+3-methylbutanoic acids	35.8[a]	8.5	30.9[a]	8.9	33.4[A]
total	**3890.2**	604.1	**3228.7**	198.5	

a, b, c, d – values with the same letters mean no significant difference at 95 % confidence level; **nd** – not detected; **A**- Ferreira *et al*., 2000; **B**- Guth, 1997; **C**- Moreno *et al*., 2005; **D**- Chaves *et al*., 2007; **E**- Boidron *et al*., 1988

Ethyl esters of organic acids are formed during wine aging by chemical esterification between alcohol and acids (Ribéreau-Gayon *et al*., 2006; Sumby *et al*., 2010). Ethyl esters of organic acids like ethyl lactate and diethyl succinate are present at higher levels in wines (Vilanova and Oliveira, 2012). However in our samples these two compounds were not detected or found in low concentrations once the wines were very young. The perception thresholds of ethyl lactate

and diethyl succinate are very high (Table 7.5) and only ethyl lactate may occasionally contribute to the wine aroma (Dubois, 1994). However esters are very important for the flavour profile of fermented beverages, the presence of different esters is often having synergistic effect, and may reduce individual perception thresholds (Dubois, 1994; Sumby *et al*., 2010).

Acetates of higher alcohols decreased slightly after MLF. Isoamyl acetate was found in all wine samples in concentrations above its perception threshold of 30 µg/L (Moreno *et al*., 2005). According to Oliveira *et al*. (2008), isoamyl acetate and 2-phenylacetate, together with the ethyl esters ethyl butyrate, ethyl hexanoate, ethyl octanoate and ethyl decanoate are the main contributors to the aroma of young wines. In our samples, these compounds, except 2-phenylacetate, were present in concentrations above their perception thresholds, indicating that the resulting wines may have "sweet" and "fruity" flavours.

The volatile phenol 4-vinylguiacol was found statistically different for wines after AF and after MLF, showing less concentration after MLF. It is known that the concentrations of 4-vinilguiacol and 4-vinylphenol use to increase after MLF, in result of the degradation of some phenolic acids by bacteria (Lerm *et al*., 2010). However, MLF may have contradictory effect on the sensory character of wine. It depends on the bacteria strain used, the presence and availability of precursors, the wine type and the vinification conditions (Lerm *et al*., 2010).

The volatile fatty acids hexanoic acid, octanoic acid and 3-methylbutanoic acid (only for wine after AF) were also found in all samples in concentrations above its perception thresholds of 420 µg/L, 500 µg/L and 33.4 µg/L, respectively (Ferreira *et al*., 2000). Although fatty acids are characterized by unpleasant notes (sweat, cheese), their flavour is essential to the aromatic equilibrium of wines (Etiévant, 1991; Vilanova and Oliveira, 2012).

7.4 Conclusions

Continuous fermentations are advantageous over batch fermentations as they have shorter fermentation times and higher productivities. Grape skins were found to be an appropriate support to be used in continuous alcoholic and malolactic fermentations. Immobilized cell systems on grape skins have an optimal mechanical stability for use in packed bed reactor in continuous mode of operation.

The integrated continuous process of winemaking gave good operational stability and promising results for further research. The obtained results on aroma compounds, suggested that the produced wines had fruit and fresh flavour. Immobilized bacteria cell system is strongly influenced by the pH value of the media and further studies in this aspect are needed. However malolactic fermentation was successful and well conducted.

7.5 References

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8. Final Remarks

The final remarks of this work are about the general conclusions that were obtained from the developed research. Moreover, some suggestions for future activities are proposed.

8.1 General Conclusions

The work presented in this thesis is the result of a systematic study that aimed at the use of immobilized cell systems for the production of wine. Overall, a high productivity process was developed that allows for the continuous production of wine, by the sequential integration of two packed bed reactors with immobilized yeast, for AF, and immobilized bacteria, for MLF, respectively.

The paragraphs below detail the main conclusions of the present work:

- Grape skins, grape stems and corn cobs are promising potential supports for yeast and bacterial cell immobilization;
- Fermentations with immobilized cells are twice as faster as fermentations with free cells;
- Fermentations with immobilized cells have higher productivities and higher fermentation rates compared to fermentations with free cells;
- The use of grape residues for yeast and bacterial cell immobilization implied in winemaking is an environmental friendly process;
- Immobilized cell system using grape skins as support form a mechanically strong/stable system that can be used for at least 10 consecutive batch fermentations;
- Immobilized bacteria and yeast cells are more protected against the negative influence of inhibitors like high ethanol and SO_2 concentrations;
- Continuous fermentations with immobilized cell systems are more beneficial than fermentations in batch mode, as the high cell population brings to higher fermentation rates and productivities;
- Grape skins are a natural material with great mechanical stability for use in batch and in continuous fermentation processes.
- The natural materials used for immobilization have an positive effect on the metabolism of the immobilized cells;
- Static batch immobilization assays proportioned higher cell load on the support, than immobilization assays with agitation;
- Yeast cells immobilized on grape skins can be stored for at least 1 month at 4 °C without losing their fermentation activities;
- Bacteria cells immobilized on grape skins grape stems or corn cobs can be stored for at least 1 month at 25 °C without losing their fermentation activities;

- Wines produced with immobilized cells on grape skins have improved quality and are not found different, in terms of sensory characteristics, from wines produced with free cells;
- The only drawback during the production of wines with cells immobilized on grape skins was found in terms of colour parameters. Wines produced with immobilized cells initially have darker colour than wines produced with free cells. However with time and with successive fermentations the colour of the wine tends to stabilize and be more similar to the colour of the control wines.

8.2 Future work

Some suggestions for future work are presented below:

- To study the relationship between pH and SO_2 and to understand the effect of these parameters on the survival of the immobilized bacteria cells;
- To evaluate eventual DNA changes in the immobilized cells throughout the process (long term operation effects on cells activity);
- To establish eventual correlations between the aroma compounds of the must and in the produced wines after integrated continuous fermentation process with immobilized and with free cells;
- To scale-up the developed integrated continuous process of winemaking.